



Aalborg Universitet

AALBORG UNIVERSITY
DENMARK

Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources

Qiu, Guanglei; Zuniga-Montanez, Rogelio; Law, Yingyu; Thi, Sara Swa; Nguyen, Thi Quynh Ngoc; Eganathan, Kaliyamoorthy; Liu, Xianghui; Nielsen, Per H.; Williams, Rohan B.H.; Wuertz, Stefan

Published in:
Water Research

DOI (link to publication from Publisher):
[10.1016/j.watres.2018.11.011](https://doi.org/10.1016/j.watres.2018.11.011)

Creative Commons License
CC BY-NC-ND 4.0

Publication date:
2019

Document Version
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

Citation for published version (APA):
Qiu, G., Zuniga-Montanez, R., Law, Y., Thi, S. S., Nguyen, T. Q. N., Eganathan, K., Liu, X., Nielsen, P. H., Williams, R. B. H., & Wuertz, S. (2019). Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources. *Water Research*, 149, 496-510.
<https://doi.org/10.1016/j.watres.2018.11.011>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

Accepted Manuscript

Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources

Guanglei Qiu, Rogelio Zuniga-Montanez, Yingyu Law, Sara Swa Thi, Thi Quynh Ngoc Nguyen, Kaliyamoorthy Eganathan, Xianghui Liu, Per H. Nielsen, Rohan B.H. Williams, Stefan Wuertz

PII: S0043-1354(18)30932-1

DOI: <https://doi.org/10.1016/j.watres.2018.11.011>

Reference: WR 14218

To appear in: *Water Research*

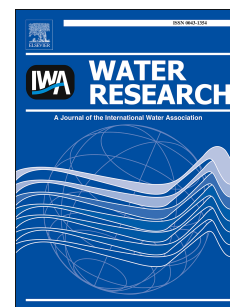
Received Date: 15 July 2018

Revised Date: 17 October 2018

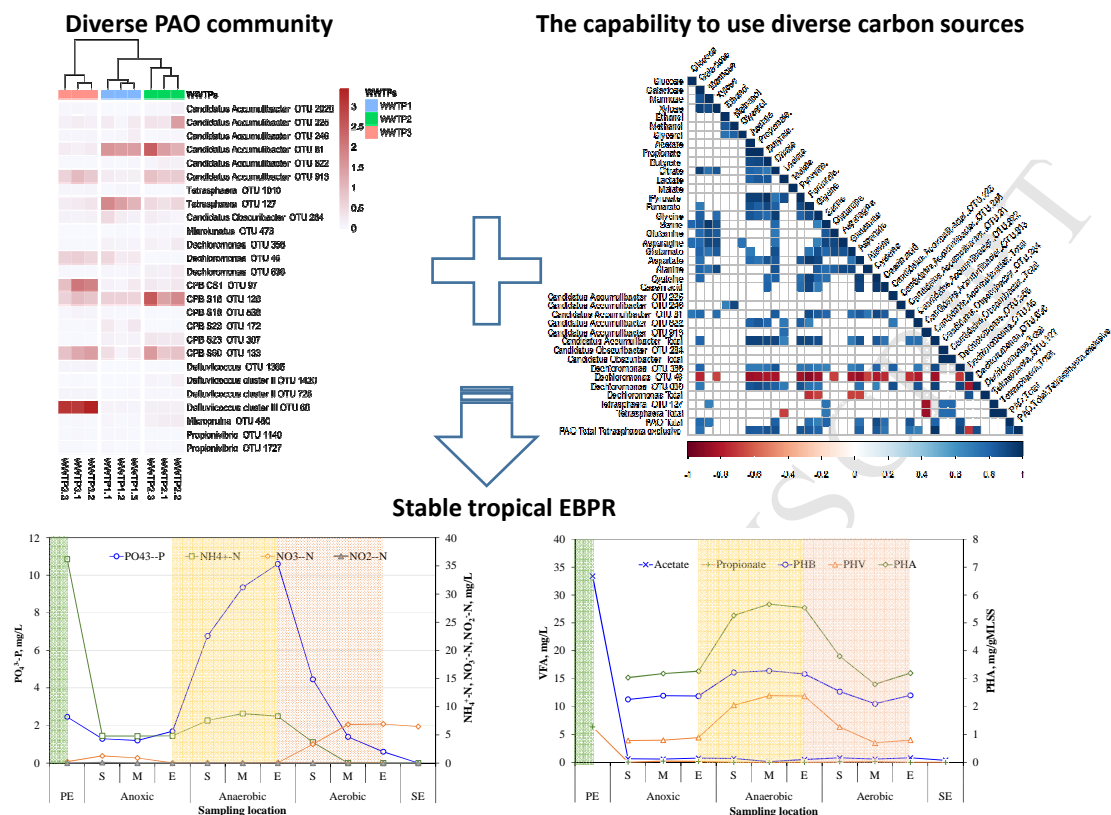
Accepted Date: 6 November 2018

Please cite this article as: Qiu, G., Zuniga-Montanez, R., Law, Y., Thi, S.S., Ngoc Nguyen, T.Q., Eganathan, K., Liu, X., Nielsen, P.H., Williams, R.B.H., Wuertz, S., Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources, *Water Research*, <https://doi.org/10.1016/j.watres.2018.11.011>.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Graphical abstract



Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources

Guanglei Qiu^{1∇*}, Rogelio Zuniga-Montanez^{1,2}, Yingyu Law¹, Sara Swa Thi¹, Thi Quynh Ngoc Nguyen¹, Kaliyamoorthy Eganathan³, Xianghui Liu¹, Per H. Nielsen^{1,4}, Rohan B. H. Williams³, Stefan Wuertz^{1,2,5 *}

¹*Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore 637551, Singapore.*

²*Department of Civil and Environmental Engineering, One Shields Avenue, University of California, Davis, California 95616, USA.*

³*Singapore Centre for Environmental Life Sciences Engineering, National University of Singapore, Singapore 119077, Singapore.*

⁴*Centre for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220, Aalborg, Denmark.*

⁵*School of Civil and Environmental Engineering, Nanyang Technological University, Singapore 639798, Singapore*

[∇]*Current affiliation: School of Environment and Energy, South China University of Technology, Guangzhou 510006, China*

Corresponding Author: swuertz@ntu.edu.sg (S.W.); qiugl@scut.edu.cn (G.Q.)

ABSTRACT

Enhanced biological phosphorus removal (EBPR) is considered challenging in the tropics, based on a great number of laboratory-based studies showing that the polyphosphate-accumulating organism (PAO) *Candidatus Accumulibacter* does not compete well with glycogen accumulating organisms (GAOs) at temperatures above 25°C. Yet limited information is available on the PAO community and the metabolic capabilities in full-scale EBPR systems operating at high temperature. We studied the composition of the key functional PAO communities in three full-scale wastewater treatment plants (WWTPs) with high in-situ EBPR activity in Singapore, their EBPR-associated carbon usage characteristics, and the relationship between carbon usage and community composition. Each plant had a signature community composed of diverse putative PAOs with multiple operational taxonomic units (OTUs) affiliated to *Ca. Accumulibacter*, *Tetrasphaera* spp., *Dechloromonas* and *Ca. Obscuribacter*. Despite the differences in community composition, ex-situ anaerobic phosphorus (P)-release tests with 24 organic compounds from five categories (including four sugars, three alcohols, three volatile fatty acids (VFAs), eight amino acids and six other carboxylic acids) showed that a wide range of organic compounds could potentially contribute to EBPR. VFAs induced the highest P release (12.0-18.2 mg P/g MLSS for acetate with P release-to-carbon uptake (P:C) ratios of 0.35-0.66 mol P / mol C, 9.4-18.5 mg P/g MLSS for propionate and P:C ratios of 0.38-0.60, and 9.5-17.3 mg P/g MLSS for n-butyrate), followed by some carboxylic acids (10.1-18.1 mg P/g MLSS for pyruvate, 4.5-11.7 mg P/g MLSS for lactate and 3.7-12.4 mg P/g MLSS for fumarate) and amino acids (3.66-7.33 mg P/g MLSS for glutamate with a P:C ratio of 0.16-0.43 mol P/ mol C, and 4.01-7.37 mg P/g MLSS for aspartate with a P:C ratio of 0.17-0.48 mol P/ mol C). P-release profiles (induced by different carbon sources) correlated closely with PAO community composition. High micro-diversity was observed within the *Ca. Accumulibacter* lineage, which represented the most abundant PAOs. The total population of *Ca. Accumulibacter* taxa was highly correlated with P-release induced by VFAs, highlighting the latter's importance in tropical EBPR systems. There was a

strong link between the relative abundance of individual *Ca. Accumulibacter* OTUs and the extent of P release induced by distinct carbon sources (e.g., OTU 81 and amino acids, and OTU 246 and ethanol), suggesting niche differentiation among *Ca. Accumulibacter* taxa. A diverse PAO community and the ability to use numerous organic compounds are considered key factors for stable EBPR in full-scale plants at elevated temperatures.

Keywords: Enhanced biological phosphorus removal (EBPR); high temperature; polyphosphate-accumulating organisms (PAO); *Candidatus Accumulibacter*; *Tetrasphaera*; carbon source; volatile fatty acids (VFAs); amino acids; sugars; alcohols

1 INTRODUCTION

Enhanced biological phosphorus removal (EBPR) remains one of the most cost-effective and sustainable processes and is widely employed in full-scale wastewater treatment plants (WWTPs) for the elimination from water and potential recovery of phosphorus (Oehmen et al., 2007; He and McMahon, 2011). However, a number of studies have shown that high temperature can be detrimental to EBPR, with PAOs being outcompeted by glycogen accumulating organisms (GAOs) at temperatures above 25°C (Whang and Park, 2002; Panswad et al., 2003; Lopez-Vazquez et al., 2009). There have been efforts to achieve stable high-temperature EBPR in the laboratory. Freitas et al. (2009) obtained robust EBPR activity at 30°C for over 100 days by applying a short cycle (consisting of a 20-min anaerobic phase and a 10-min aerobic phase), and up to 100% P removal efficiency was achieved in an aerobic granular biomass system by selectively removing sludge from the top of the sludge bed (Winkler et al. 2011). Recently, long-term EBPR stability has been

demonstrated in lab-scale reactors without applying short SRTs or selective sludge removal (Ong et al., 2014). Using acetate as a sole carbon source, the predominant PAO in the reactor was *Candidatus Accumulibacter* clade IIF. However, as the temperature increased from 24 to 32°C, EBPR activities were compromised, with a significant reduction in the relative abundance of *Ca. Accumulibacter* and a concurrent increase in the GAO population. Shen et al. (2017) further showed that having multiple anaerobic/aerobic stages in one EBPR cycle helped to achieve EBPR at 30°C with either acetate or propionate as carbon sources. Acetate resulted in higher process stability as compared to propionate. These laboratory-scale studies suggested the need for special operational controls to allow PAOs to outcompete GAOs at elevated temperature. In contrast, EBPR has been observed in a full-scale activated sludge plant in Singapore with year-round operation at 28-32°C, although the plant was not designed for EBPR. Both GAOs and PAOs were present, but did not seem to compete with one another (Law et al., 2016). Clearly, there are remaining knowledge gaps between lab-scale studies and full-scale observations for EBPR at high temperatures. Apart from the study from Law et al (2016), very limited information is available on the key functional PAOs and their metabolic characteristics in full-scale EBPR systems under tropical conditions.

Among the many parameters governing EBPR viability and stability, the type of carbon source is key (Abu-Gharach and Randall, 1991; Shen and Zhou 2016). It is also one of the main differences between lab-scale and full-scale systems. To date, short-chain volatile fatty acids (VFAs) are the most commonly used carbon substrates in lab-scale EBPR systems. Yet PAOs consist of diverse members that can metabolise different substrates. *Ca. Accumulibacter* is commonly found in lab-scale and full-scale EBPR systems (Seviour et al., 2003; Lu et al., 2006; Fukushima et al., 2007; Oehman et al., 2007; Ong et al., 2014; Shen et al., 2017; Rubio-Rincón, et al., 2017), and primary carbon sources are restricted to low molecular weight substrates such as VFAs (Oehman et al., 2007; He and McMahon, 2011; Flowers et al., 2013). Another important group of PAOs is the actinobacterial *Tetrasphaera* spp. (Hanada et al., 2002; Kong et al., 2005). They are more abundant

than *Ca. Accumulibacter* in WWTPs in countries with lower wastewater temperatures, such as Denmark (9-18°C, Saunders et al., 2016; Stockholm-Bjerregaard et al., 2017), Portugal (8-25°C, Lanham et al., 2013) and Poland (5-24°C, Muszyński and Załęska-Radziwiłł, 2015), as well as in some full-scale and pilot-scale MBR plants in The Netherlands, Norway, Germany and Switzerland (Silva et al., 2012), accounting for up to 40% of the biomass. In contrast to *Ca. Accumulibacter*, *Tetrasphaera* spp. are more versatile in substrate uptake capabilities, can utilise both glucose and amino acids (Nguyen et al., 2011; Nguyen et al., 2015; Marques et al., 2017), and are capable of fermenting complex organics (Nielsen et al., 2010; Kristiansen et al., 2013; Marques et al., 2017) and accumulating fermentation by-products (Nguyen et al., 2015). Some were shown to be VFA users, but in situ staining did not support that these probe-defined *Tetrasphaera* store intracellular polyhydroxyalkanoate (PHA) (Kong et al., 2005; Nguyen et al., 2011). Carbon usage characteristics are an important trait that differentiates these two groups of PAOs. Apart from *Ca. Accumulibacter* and *Tetrasphaera* spp., other putative PAOs have been identified in EBPR systems, some occurring in full-scale plants in appreciable numbers (Stokholm-Bjerregaard et al. 2017), including *Microlunatus phosphovorus* (Nakamura et al., 1995), *Ca. Accumulimonas* (Nguyen et al., 2012), *Dechloromonas* (Kong et al., 2007), *Ca. Obscuribacter* (Soo et al., 2014), *Thiothrix caldifontis* (Rubio-Rincon et al., 2017) and Comamonadaceae members (Ge et al., 2015), among others. Dissimilarity was also observed in their carbon metabolism; for example, the actinobacterium *Microlunatus phosphovorus* showed a similar metabolism to that of *Tetrasphaera*. It utilises a wide range of sugars and amino acids, but takes up acetate slowly (Nakamura et al., 1995; Ubakata and Takii, 1998). Genome analysis suggested the lack of *phaABC* genes for PHA synthesis (Kawakoshi et al., 2012). Gammaproteobacterial *Ca. Accumulimonas* was shown to take up VFAs and store them as PHAs, similar to *Ca. Accumulibacter* (Nguyen et al., 2012).

Municipal wastewater contains a complex matrix of organic compounds. It is logical to conceive that other carbon sources, apart from VFAs, and other PAOs, apart from *Ca. Accumulibacter*, could

contribute to EBPR in full-scale plants. In this study, repeated field sampling was carried out in three tropical WWTPs in Singapore to monitor the EBPR activity. The objectives were to (i) characterise the bacterial and PAO community using 16S rRNA amplicon sequencing combined with fluorescent in situ hybridisation (FISH) and poly-P chemical staining; (ii) characterise the carbon utilisation profiles of these functional PAOs, by subjecting fresh activated sludge to anaerobic P-release tests with 24 carbon sources from five categories (including four sugars, three alcohols, three VFAs, eight amino acids and six carboxylic acids); and (iii) explore the associations between P-release induced from the utilisation of different carbon sources and the bacterial community. We hypothesised that differential utilisation of carbon sources is associated with a distinct PAO community. Six of the carbon sources were further selected for anaerobic-aerobic cycle studies to evaluate their effectiveness in supporting EBPR.

2 MATERIALS AND METHODS

2.1 Sampling of wastewater treatment plants

Field sampling was conducted at three domestic WWTPs with different configurations in Singapore from November 2016 to February 2017 (Supplementary Table S1). WWTP1 has four identical treatment trains using a 5-stage step-feeding activated sludge process, where mixed liquor passes through five basins of alternating anoxic and aerobic zones with the influent equally distributed into the anoxic zones of each basin. WWTP2 consists of six trains of activated sludge treatment, each one featuring a modified Ludzack–Ettinger (MLE) configuration. WWTP3 has three parallel treatment trains, each comprising an anoxic tank followed by an anaerobic and an aerobic tank and, finally, by a membrane tank for solid-liquid separation (Table 1 and Supplementary Fig. S1). Water temperature at these plants varies from 28.7 to 31.6°C.

Field sampling was performed in three episodes at each plant to monitor process performance and the bacterial community composition. Liquid samples were collected from the primary effluent and at different locations of each plant (Supplementary Fig. S1). Temperature, pH and dissolved oxygen (DO) were measured with a multi-parameter portable meter (YSI Professional Plus, CA, USA) at each sampling point. The mixed liquor samples were filtered through 0.22- μ m sterile filters for soluble chemical oxygen demand (SCOD), NH_4^+ -N, NO_3^- -N, NO_2^- -N, PO_4^{3-} -P and VFA analyses. Non-filtered primary effluent samples were acidified with sulphuric acid and analysed for total phosphorus (TP) and total chemical oxygen demand (TCOD). Mixed liquor samples were collected from each sampling location and fixed with 2 drops of 37% formaldehyde for PHA and glycogen analyses. For the microbial community analysis, 2 ml of mixed liquor were collected from the end of the aerobic zones, snap-frozen in liquid nitrogen and stored at -80°C before DNA extraction. For FISH imaging, activated sludge was collected from the end of the anaerobic/anoxic zones and the end of the aerobic zone, and immediately fixed using paraformaldehyde (PFA at a final concentration of 4%, for Gram-negative bacteria) and ethanol (by mixing equal volumes of 100% ethanol and mixed liquor, for Gram-positive bacteria). Fresh mixed liquor was also collected at the end of the aerobic zones for anaerobic P-release tests and anaerobic-aerobic cycle studies.

2.2 EBPR activity tests with different carbon sources

For anaerobic P-release tests, the mixed liquor was diluted with secondary effluent from the corresponding WWTPs that was depleted of NO_2^- and NO_3^- to a final MLSS of 2.0 g/L; the solution pH was adjusted to 7.25 using 0.1M HCl or 0.1M NaOH. Fifty millilitres of diluted activated sludge were added into 50-ml culture bottles and sealed. Anaerobic conditions in each bottle were induced by N_2 gas purging for 15 min before the different carbon sources were added. Twenty-four carbon sources were tested, including four sugars: glucose, galactose, mannose, and xylose; three alcohols: ethanol, methanol and glycerol; nine amino acids: glycine, serine, glutamine, asparagine, glutamate, aspartate, alanine, cysteine and casein acid; three VFAs: acetate, propionate and n-butyrate; and five

carboxylic acids: citrate, lactate, malate, pyruvate and fumarate. Each bottle received one carbon source at a final COD concentration of 300 mg/L, and the culture bottles were placed for 3 h in a shaking incubator (Infors HT, Bottmingen, Switzerland) operated at 180 rpm and 30°C. Samples were collected every hour and passed immediately through 0.45-µm sterile filters for $\text{PO}_4^{3-}\text{-P}$ analysis. One culture bottle with activated sludge but no carbon source addition served as a control. All the experiments were done in duplicates.

Glucose, methanol, glutamate, aspartate, acetate, and propionate were further selected for anaerobic-aerobic cycle studies. Experiments were performed in 1-L reactors operated in parallel in a 30°C water bath. Fresh activated sludge was diluted to a final MLSS of 2.0 g/L, followed by the addition of each carbon source to a final concentration of 30 mg C/L. Fresh primary effluent was used as one of the treatments, acting as a positive control. A reactor with activated sludge but no carbon source addition served as a negative control. The full cycle consisted of a 3-h anaerobic phase followed by a 3-h aerobic phase. N_2 gas and air were purged continuously during the anaerobic and aerobic phases, respectively, to ensure anaerobic and aerobic conditions and for mixing. The solution pH was maintained at 7.25 ± 0.05 during the experiment by addition of 0.25 M HCl or 0.1 M NaHCO_3 . Filtered water (through 0.45µm membrane filters) and activated sludge samples were collected at different time intervals for $\text{PO}_4^{3-}\text{-P}$, VFAs, total organic carbon (TOC), PHA and glycogen analyses.

2.3 Analytical methods

MLSS and MLVSS were determined according to Standard Methods (APHA, 1999). COD, $\text{NH}_4^+\text{-N}$, TP, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{PO}_4^{3-}\text{-P}$ were measured using test kits (HACH, CO, USA) following Standard Methods (APHA, 1999). TOC and TN were analysed using a TOC/TN analyser (Shimadzu, Japan), and VFAs (acetate, propionate and butyrate) were measured using a gas chromatograph (Prominence, Shimadzu, Japan) equipped with a flame ionisation detector that was fitted with a DB-FFAP column (30 × 0.25 mm) (Agilent Technology, U.S.). PHA analyses were performed according to Oehmen et

al. (2005a), using a gas chromatograph (Prominence, Shimadzu, Japan) equipped with an FID detector and fitted with a DB-5MS Ultra Inert column (30×0.25 mm) (Agilent Technology, CA, USA). Glycogen analyses were carried out by measuring glucose in the sludge after acid digestion as described by Kristiansen et al. (2013).

2.4 Fluorescence in situ hybridisation (FISH)

PFA-fixed activated sludge samples were washed with 1× phosphate-buffered saline (PBS) solution and resuspended in a mixed solution of 1×PBS and 100% ethanol (50:50). Washed PFA-fixed and ethanol-fixed samples were stored at -20°C before FISH analysis. Organisms of interest were detected using EUB probe mix, targeting most Bacteria (EUB338, EUB338II and EUB338III) (Daims et al., 1999), and PAOmix (PAO651, PAO462 and PAO846) (Crocetti et al., 2000) and Tetmix (Tet1–266, Tet2–174, Tet2–892 and Tet3–654) (Nguyen et al., 2011), targeting *Ca. Accumulibacter*- and *Tetrasphaera*- PAOs, respectively. Efforts were made to visualise the two groups of PAOs simultaneously using samples fixed with either PFA or ethanol. PFA-fixed samples allowed for good detection of both *Ca. Accumulibacter* and *Tetrasphaera*. In contrast, low detection of *Ca. Accumulibacter* was observed with ethanol-fixed samples. Thus, PFA-fixed samples were used for FISH image generation in this study.

2.5 DNA extraction, 16S rRNA gene amplicon sequencing and qPCR

Genomic DNA was extracted using the Fast DNATM 2 mL SPIN Kit for Soil samples (MP Biomedicals, CA, USA), following the optimised protocol for activated sludge (Albertsen et al. 2015). Bacterial 16S rRNA gene amplicon sequencing was performed, targeting the V1-V3 region (primer set: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG). PCR amplification was carried out in a 25-µl PCR matrix containing 10 ng of genomic DNA, 400 nM dNTPs, 1.5 mM MgSO₄, 2 mU Platinum R Taq DNA polymerase high fidelity, 1× Platinum R High Fidelity buffer (Thermo Fisher Scientific, MA, USA) and a pair of barcoded library adaptors (400

nM), with a thermo cycler setting of initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and final elongation at 72°C for 5 min. All PCR reactions were run in duplicate and pooled afterwards. The amplicon libraries were purified using the Agencourt R AMpure XP bead protocol (Beckmann Coulter, CA, USA) with 1.8 bead solution/PCR solution ratio. Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations. The library pool was sequenced on a MiSeq (Illumina, CA, US) using a MiSeq Reagent kit v3 (2×300 paired end). Pre-processing of all amplicon libraries was performed according to Albertsen et al. (2015). Taxonomy was assigned using MiDAS v.1.20 (McIlroy et al. 2015).

Additionally, quantitative PCR (qPCR) was used to analyse the clade level distribution of *Ca. Accumolibacter* in each plant, according to He et al. (2007).

2.6 Statistical analysis

All statistical analyses were performed using SPSS 13 (IBM, NY, USA) or R Version 3.3.33 (www.r-project.org). The heat-map of the community compositions was plotted using the R package pheatmap Version 1.0.8 (<https://cran.r-project.org/web/packages/pheatmap>). Complete linkage clustering analysis was based on Euclidean distances. Pearson tests were performed using the R package Hmisc Version 4.1-1 (<https://cran.r-project.org/web/packages/Hmisc>) to examine the correlation between the P-release values obtained with the different carbon sources and the relative abundance of putative OTUs belonging to PAOs in each WWTP. The plot of the obtained Pearson correlation coefficient matrix was conducted with the R package Corrplot Version 0.84 (<https://cran.r-project.org/web/packages/corrplot>), with a P-value cut-off of <0.05. Raw P-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Regularised canonical correlation analysis (CCA) was performed using the R package CCA Version 1.2 (<https://cran.r-project.org/web/packages/CCA>) to study the inter-relationships between the relative abundance of bacterial taxa and P-release resulted from different carbon sources. The Venn

diagram of the community compositions was plotted using the online tool VENNY Version 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

3 RESULTS AND DISCUSSION

3.1 In-situ EBPR and nutrient removal activities

During the nine sampling events conducted at three WWTPs, high EBPR activity was observed together with good nitrogen removal (Fig. 1). We recorded P-release at the end of the anoxic/anaerobic zones concomitant with an increase in the intracellular PHA content of the activated sludge. In the subsequent aerobic phase, P-uptake coincided with a decrease in PHA content, resulting in low $\text{PO}_4^{3-}\text{-P}$ concentrations at the end of the aerobic stages ($< 2.0 \text{ mg/L}$). Neither WWTP1 nor WWTP 2 has a defined anaerobic stage (Supplementary Fig. S1); however, P-release and denitrification occurred simultaneously within the same compartment. We surmise that as long as the wastewater had sufficient carbon for both denitrification and EBPR, a separation of the anoxic and anaerobic zones was not necessary, as non-denitrifying PAOs were able to perform anoxic-aerobic EBPR by recognising anoxic conditions as anaerobic (Cokro et al. 2017). For WWTP1, the highest P-release was observed in the first anoxic stage (Fig. 1A), followed by a decrease in subsequent stages due to the dilution of the primary effluent with the mixed liquor along the treatment train, and carbon consumption via denitrification. This outcome agrees with results obtained in a lab-scale EBPR system with multiple anaerobic/aerobic stages in one SBR cycle, where decreasing P-release was observed along multi-stages (Shen et al., 2017). Additionally, a significantly lower PHA content was observed at WWTP1 compared to WWTPs 2 and 3, likely resulting from a faster carbon turnover in the step-feed system (Shen et al., 2017). For all plants, polyhydroxybutyrate (PHB) was the major PHA polymer in the sludge, reflecting that acetate was the main VFA present in the primary effluent, followed by much lower concentrations of propionate.

Low in-tank VFA concentrations (<1.0 mg/L) were observed within all three treatment trains, which is considered beneficial for the prevalence of PAOs. These organisms rely on the proton motive force generated by the hydrolysis of poly-P and the resultant efflux of proton in symport of phosphate for VFA uptake; hence they are more efficient in scavenging VFAs at low concentrations than *Defluviicoccus*-GAOs (Burow et al., 2008; Tu and Schuler, 2014).

3.2 Bacterial community composition

The bacterial communities in the three WWTPs were analysed using 16S rRNA gene amplicon sequencing. At the phylum level, all the communities were dominated by Proteobacteria (with relative abundances from 37 to 48%), primarily members of the Beta- (10-19%), Alpha- (9.5-21%) and Gamma- (3.7-7.3%) classes, and followed by Bacteroidetes (14-23%), Actinobacteria (11-19.1%), Chloroflexi (7.2-19%) and Firmicutes (2.0-3.2%) (Fig. 2A). All these phyla are common in activated sludge communities (Albertsen et al., 2015), and the relative abundance of other bacterial phyla was below 2.5%.

High community similarity was observed for samples from the same plant (>85%, Supplementary Table 1), whereas communities from different plants showed higher degrees of disparity (similarity values ranged from 29-75%). At each plant, around half (44.3-60.9%) of the OTUs with at least three reads in each library were consistently detected during the three sampling episodes. About 25% of all OTUs were detected at least once across all the plants (Fig. 2C), including almost all the putative PAO- and GAO-related OTUs. This confirms that systems performing EBPR contain a core community of functional microorganisms (Saunders et al., 2016).

A variety of sequences related to PAOs and GAOs were identified in the nine amplicon libraries (see Fig. 2B, where only taxa with a relative abundance >0.02% are listed). For PAOs, six *C. Accumulibacter* OTUs occurred in all three plants; WWTP2 showed the highest relative abundance (six OTUs, 2.6-3.8%), followed by WWTP1 (five OTUs, 1.70-2.04%) and WWTP3 (six OTUs,

0.95-1.40%). The observed relative abundance values of *Ca. Accumulibacter* fell in the lower range of those found in full-scale plants in temperate countries, accounting for 2-22% as quantified by FISH imaging (Kong et al., 2004; Chua et al., 2006; Gu et al., 2008; Lopez-Vazquez et al., 2008; Zhang et al., 2011; Mielczarek et al., 2013), 1.2-24% by qPCR (He et al., 2007; He et al., 2008; Mao et al., 2015; Zhang et al., 2016), 0.5-10% by 16S rRNA gene amplicon sequencing (Law et al., 2016; Saunders et al., 2016; Stokholm-Bjerregaard et al., 2017) and 4.8% by metagenomics (Albertsen et al., 2012). All the *Ca. Accumulibacter* taxa were likely type II, except for OTU 2020, which differs from typical type I or type II sequences (Supplementary Table 2, Flowers et al., 2009). Based on qPCR results, IIB and IIC were the predominant clades in the WWTPs (Supplementary Table 3). In comparison, clades IIA, IIB and IIC were abundant in full-scale WWTPs in the U.S. (He et al. 2007), and IIC and IID were the most dominant clades in 18 full-scale WWTPs from six countries (Mao et al. 2015). Further, clades IA, IIB and IIC were consistently detected in eight geographically and operationally distinct WWTPs (Zhang et al. 2017). Overall, it appears that clades IIB and IIC are dominant in both temperate and tropical regions.

Two *Tetrasphaera* OTUs were detected, but only one (OTU127) was dominant in all three WWTPs. Total *Tetrasphaera* OTUs were most abundant in WWTP1 (1.1-1.8%), followed by WWTP2 (0.37-0.55%) and WWTP3 (0.23-0.37%) (Fig. 2B). Phylogenetic analysis of the 16S rRNA gene of the two *Tetrasphaera* OTUs suggested both of them are closely related to clade 3 (Nguyen et al., 2011). Both *Ca. Accumulibacter* and *Tetrasphaera* were detected by FISH in all three plants (Fig. 3). Almost all the *Ca. Accumulibacter* cells and a large proportion of *Tetrasphaera* cells accumulated P as indicated by FISH and poly-P dual-staining (Supplementary Fig. S2), suggesting that they are active PAOs in the plants. Other putative PAOs, e.g. *Ca. Obscuribacter* (0.06-0.49%) and *Microlunatus* (1 OTU, 0.01-0.21%) were present in minor amount. FISH and poly-P dual-staining confirmed P accumulation by other cells that were neither *Ca. Accumulibacter* nor *Tetrasphaera* (Supplementary Fig. S2).

Ca. Competibacter and *Defluviicoccus* were the dominant GAOs (Fig. 2B). Six *Ca. Competibacter*-related OTUs were detected across the nine samples, with the lowest relative abundance (0.87-1.42%) observed in WWTP1. WWTP2 and WWTP3 showed similar relative abundance of 2.55-4.58% and 2.40-4.35%, respectively; however, there was a distinct community structure at the OTU level (Fig. 2B). Nine *Defluviicoccus*-related OTUs were detected, where none of the OTUs showed a relative abundance >0.1% in any of the samples, except for *Defluviicoccus* cluster III OTU 60, which consistently occurred at high relative abundance (3.04-3.43%) in WWTP3. Cluster III *Defluviicoccus* members have a filamentous morphology and a GAO-phenotype (McIlroy et al., 2010). The relative abundance of *Defluviicoccus* in WWTP1 (0.08-0.11%) and WWTP2 (0.27-0.33%) was much lower. *Propionivibrio*- and *Micropruina*- related GAOs were detected but accounted for very minor fractions of each community. *Micropruina* showed the highest relative abundance at 0.13-0.21% in WWTP2.

The genus *Dechloromonas* was moderately abundant in all plants (0.30-0.68%, 0.20-0.48% and 0.54-0.68% for WWTPs 1, 2 and 3, respectively). OTU 46 predominated in WWTPs 1 (0.2-0.53%) and 3 (0.51-0.63%), and OTU 636 (0.11-0.23%) and OTU356 (0.08-0.22%) dominated in WWTP2. The *Dechloromonas* genus is closely related to *Ca. Accumulibacter* in the Rhodocyclaceae family. Some taxa may be PAOs, as an in-situ study showed that they behave similarly to *Ca. Accumulibacter* in terms of substrate uptake and storage of PHAs (Kong et al., 2007). They may assimilate both acetate and amino acids anaerobically (McIlroy et al. 2016) or assimilate acetate and store PHA, but without poly-P cycling (Ahn et al., 2007; Günther et al., 2009). Therefore, the *Dechloromonas* OTUs observed in this study may represent PAOs, GAOs or neither.

Overall, PAOs were more abundant (3.23-4.33%) than GAOs (1.02-1.74%) in WWTP1, while in WWTP2, the abundances of PAOs (3.19-4.51%) and GAOs (3.04-5.05%) were similar. GAOs (4.26-4.70%) were much more abundant than PAOs (1.30-1.78%) in WWTP3. All three plants contained a diverse EBPR community composed of a rich collection of PAOs and GAOs with many OTUs. Most

of the OTUs were detected across plants but with distinct relative abundances, suggesting that each plant had its own signature EBPR community (Fig. 2B).

3.3 Anaerobic P-release profiles with different carbon sources

Systematic anaerobic P-release tests were performed on the activated sludge samples obtained from the three WWTPs, using 24 carbon sources from five categories representing the most commonly found hydrolysates of the three major groups of organic matter: carbohydrates, lipids, and proteins (Nielsen et al., 2010). All samples had PAOs capable of using a wide range of compounds and despite the disparity in PAO community structures, similar P-release patterns were observed across all plants (Fig. 4). VFAs resulted in the highest P-release of 24.0-39.4, 18.8-37.0 and 17.0-34.6 mg/L for acetate, propionate and butyrate, respectively, with an MLSS of 2 g/L. The values were comparable to those obtained with acetate in temperate EBPR plants, such as 8-15 mg P/g SS in 28 Danish WWTPs (Mielczarek et al., 2013), 5.1-24.3 mg P/g VSS in six WWTPs in the United States (Gu et al., 2008), and up to 15.8 mg P/g SS in ten EBPR plants in China (Zhang et al., 2011). Substantial P release was observed for some carboxylic acids (i.e., 20.2-36.2 mg/L for pyruvate, 9.0-23.4 mg/L for lactate and 7.4-24.8 mg/L for fumarate), which constituted the second most efficient group of carbon sources, following VFAs. This result was not unexpected since pyruvate and fumarate are important intermediates in the anaerobic carbon metabolism of *Ca. Accumulibacter* and *Tetrasphaera* (He and McMahon, 2011; Kristiansen et al., 2013). All nine amino acids resulted in substantial P-release (up to 23.2 mg/L); values were comparable to those in Danish WWTPs (where high P-release of up to 12.0 mg P/g VSS was obtained with glycine), despite the much lower relative abundances of *Tetrasphaera* and *Ca. Accumulibacter* in our study. Relatively low P-release was observed for sugars, alcohols and other carboxylic acids (e.g. malate). These findings highlight the importance of VFAs in tropical EBPR systems, as generally understood for temperate EBPR (Oehmen et al., 2007), but at the same time underscores the potential roles of other compounds (e.g.,

pyruvate, fumarate, lactate, amino acids and in some cases, glucose), which could serve as complementary carbon sources.

The P-release values induced by each carbon source were normalised against the P-release from the acetate treatment and from the same activated sludge source, and a clustering analysis was performed (Fig. 5). Interestingly, the P-release profiles corresponding to the utilisation of different carbon sources for activated sludge from each plant clustered together, suggesting a connection between carbon usage characteristics and PAO community composition.

3.4 Relating carbon source usage to bacterial/PAO community composition

If certain taxa in the activated sludge preferentially do utilise specific carbon sources for P-release, then these associations may be detectable by examining correlation statistics between P-release and the relative abundance of OTUs across samples, under the assumption that a greater abundance of the relevant genes will be associated with an increase in P-release magnitude. Among the top 89 most abundant bacterial OTUs that had no missing values, PAOs tended to strongly correlate with P-release associated with different carbon sources (Supplementary Fig. S3). We note that other non-PAO taxa showed a comparable degree of correlation, which could result from similar mechanistic association with carbon utilisation in a non-EBPR context, mechanistic associations with PAOs unrelated to carbon source utilization, OTUs that represent cryptic PAO species or false positive correlations associated with the sample sizes employed here. Interestingly, GAOs showed a tendency to be anti-correlated with the P-release profiles (induced from the utilisation of different carbon sources), consistent with an underlying competition with PAOs (Oehmen et al., 2007). Overall, there was a positive correlation between P-release and the PAO community.

For different carbon sources, strong positive correlations were observed for compounds within the same category (Fig. 6A), suggesting that similar compounds were processed via similar metabolic pathways and/or by specific groups of bacteria. Among categories, positive correlations were

observed between sugars and amino acids, which suggests that bacteria that can use amino acids may also be able to use sugars (Fig. 6A). *Tetrasphaera* is known to have the ability to metabolise sugars and amino acids (Kong et al., 2005; Nguyen et al., 2011; Kristiansen et al., 2013; Nguyen et al., 2015; Marques et al., 2017). Additionally, positive correlations were observed between some VFAs and amino acids, particularly, between aspartate and all the VFAs and between acetate and glutamate.

Strong positive correlations (Pearson correlations >0.91 with $P<0.001$) were observed between the total population of *Ca. Accumulibacter* and all VFAs, pyruvate and fumarate (Fig. 6A), underscoring the role of VFAs as primary carbon sources utilised by *Ca. Accumulibacter* (Oehmen et al., 2007; Flowers et al., 2013). Interestingly, strong positive correlations were also observed between the total population of *Ca. Accumulibacter* and some amino acids (aspartate, glutamate, glycine and casein acid). As also suggested by the CCA analysis (Fig. 6B), this correlation was most prominent for *Ca. Accumulibacter* OTU 81 (the most abundant *Ca. Accumulibacter* OTU in WWTP1 and the second most abundant *Ca. Accumulibacter* OTU in WWTP2), indicating a significant role in amino acid-induced P-release. Via MAR-FISH analysis, Kong et al. (2004) showed that PAO651-defined *Ca. Accumulibacter* from three full-scale WWTPs in Denmark could assimilate glutamate, but not other amino acids tested. None of the *Ca. Accumulibacter* cells from these three full-scale WWTPs in Denmark that were targeted by the PAOmix probe could use glycine (Nguyen et al. 2015). Most of the *Ca. Accumulibacter* cells from four full-scale WWTPs in Japan that hybridised with the probe could take up glutamate and aspartate anaerobically (Chua et al. 2006). Metatranscriptomic characterisation of an enrichment culture of *Ca. Accumulibacter* clade IIC strain UW-1 showed the expression of genes involved in anaerobic glycine metabolism, and P-release associated with anaerobic glycine was further demonstrated in a batch test (Oyserman et al., 2016). Using FISH and poly-P dual staining, we observed that a greater number of *Ca. Accumulibacter* cells released their poly-P after anaerobic incubation with glutamate or aspartate, suggesting they are capable of using these carbon sources anaerobically. At the same time, a number of *Ca. Accumulibacter* cells retained

their poly-P even after anaerobic incubation with glutamate or aspartate, indicating they are not efficient glutamate/aspartate users (Supplementary Fig. S2). These observations highlight that *Ca. Accumulibacter* taxa have different affinities toward amino acids and might explain the differentiation of the amino acid-using *Ca. Accumulibacter* (OTU 81) from other taxa in the correlation test (Fig. 6).

The relative abundance of *Ca. Accumulibacter* OTU 246 was closely related to alcohol-induced P-release. By comparison, a previous metagenomic study revealed two *Ca. Accumulibacter* taxa from clade IIF with a set of genes encoding the necessary enzymes to convert ethanol into acetate, which might enable them to use ethanol for EBPR (Skenner et al. 2013). Based on a comparison with type I and type II FISH probes (Flowers et al., 2009), the less abundant *Ca. Accumulibacter* OTU 246 (0.01-0.16%) in the plants sampled in this study can most likely be assigned to type II (Supplementary Table 2). Given that it was the only OTU related to alcohols among all the putative PAOs, its ability to use alcohols should be further studied.

Through CCA analysis (Fig. 6B), we observed that the predominant *Tetrasphaera* OTU 127 was positively related to the P-release induced by amino acids (asparagine, serine), sugars (glucose, mannose), malate and all the alcohols. However, except for serine and mannose, none of these correlations were significant ($P < 0.05$) in the Pearson correlation test (Fig. 6A). It is possible that the capability of some *Ca. Accumulibacter* taxa like OTU81 to use amino acids weakened the statistical relationship between *Tetrasphaera* and amino acids. In addition, a study suggested that some *Tetrasphaera* members might be able to achieve anaerobic P-uptake with sugars and/or amino acids, due to energy generation through fermentation of the carbon sources (Marques et al., 2017). This ability would mask the relationship between P-release and their population.

Ca. Obscuribacter (0.05-0.49%) showed no correlation between P-release and any of the carbon sources tested (Fig. 6A), and was only weakly connected to the same group of carbon sources that

correlated with *Tetrasphaera* (Fig.6B). *Ca. Obscuribacter* is a putative PAO predicted to be able to utilise VFAs, glucose and amino acids (Soo et al., 2014), although these features need to be further confirmed. Among the three *Dechloromonas* OTUs, OTU 46 showed significant anti-correlation with a wide range of carbon sources, including most VFAs, sugars and amino acids. Meanwhile, the other 2 OTUs (OTU 356 and 636) correlated with these compounds, but it is possible that correlations were the result of the positive correlation between total Ca. *Accumulibacter* and OTU_356 and OTU_636. Hence we are unable to unambiguously assign *Dechloromonas* OTUs to the P-release stimulated by any of the carbon sources. *Dechloromonas* was the third most abundant group of putative PAOs in the three plants, so it is necessary to further investigate their putative roles in EBPR systems. However, amplicon analysis may overestimate the relative abundance of some *Dechloromonas* OTUs as much as 10-fold compared to FISH-based quantification due to a high copy number of the 16S rRNA gene (McIlroy et al., 2016). None of the available FISH probes hybridised with the few *Dechloromonas* OTUs in our sludge samples.

3.5 Performance of selected carbon sources for EBPR

Anaerobic-aerobic cycle studies were performed with selected carbon sources (glucose, methanol, glutamate, aspartate, acetate and propionate) at a concentration of 30 mg C/L (Fig. 7). Acetate, propionate and wastewater (i.e., primary effluent) showed similar P-release profiles, resulting in high P-release at the end of the anaerobic stage. The greatest P-release was observed for WWTP2 (Fig. 7B) and corresponded with the highest relative abundance of PAOs (especially, *Ca. Accumulibacter*) (Fig. 2B). Substantial P-release was observed for glutamate and aspartate, with very similar profiles that were almost constant throughout the anaerobic operation, but at much lower rates when compared to wastewater and VFAs. Interestingly, an initial adaptation period seemed to be necessary for glucose or methanol to stimulate a substantial P-release. It is possible that these compounds were first fermented by *Tetrasphaera* or other bacteria, and that some fermentation products induced P-release by PAOs (Nielsen et al., 2010; Kristiansen et al., 2013; Marques et al., 2017). However, during the

anaerobic phase, no VFA formation was observed for any of the carbon sources and neither was significant PHA formation detected in the sludge (Supplementary Fig. S3). The lack of observable VFA formation is reasonable given that the uptake rate of VFAs is higher than that of other carbon sources (Supplementary Fig. S3). No PHA detection suggests that these carbon sources may have been converted into other intracellular storage compounds.

In the subsequent aerobic stage, substantial P-uptake was observed for all carbon sources. Some carbon sources (glutamate and methanol) allowed aerobic P-uptake without the necessity for anaerobic conversion of these carbon sources to known intracellular storage compounds (e.g., PHAs, Fig. S4). Acetate and wastewater had the highest P-uptake rates. Significantly lower P-uptake was observed for propionate. This lower P-uptake is likely due to the need for PAOs to acclimate to the different intracellular storage polymer content (higher in PHV and PH2MV). Since acetate was the major VFA in these plants, the lower P-uptake observed for propionate was not unexpected. A similar explanation might apply to other carbon sources. Despite the distinct PAO community composition among plants, glutamate always showed lower P-uptake compared to aspartate. In lab-scale reactors, aspartate and glutamate were suggested to be favoured by *Ca. Accumulibacter* and *Tetrasphaera*, respectively (Fukushima et al., 2007; Zengin et al., 2011). However, the consistent behaviour of glutamate and aspartate in the present work, across three plants, suggests that glutamate and aspartate were probably used by the same group of PAOs.

Stoichiometric values were calculated for P and carbon transformations and compared to published model values (Table 2) for acetate, propionate and wastewater; model values for other carbon sources were not available. Our stoichiometric values differ somewhat from the published ones for *Ca. Accumulibacter* (or a mixed PAO-GAO community), but generally fall within the range obtained from full-scale sludge studies in temperate regions. Aerobic stoichiometry is expected to show a higher degree of variability, since the production of biomass, polyphosphate, and glycogen from PHA can proceed independently (Smolder et al., 1995). In general, higher aerobic P-uptake/PHA-

consumption (P/PHA) values were observed in all three plants, which is in line with the observation of Lanham et al. (2013).

Among plants, the highest anaerobic P-release/VFA-uptake (P/VFA) ratios and aerobic P/PHA ratios were observed in WWTP2 samples, corresponding to the high relative abundance of *Ca. Accumulibacter*. WWTP1 had similar ratios to WWTP3, but also the lowest number of GAOs (*Ca. Competibacter*, 0.87-1.42%; *Defluviicoccus*, 0.38-0.69%). Apart from the fact that GAOs take up VFAs, which results in lower P/VFA values, different groups of PAOs have been found to show distinct stoichiometric ratios. Welles et al. (2015) suggested that, when poly-P is not limiting, type II *Ca. Accumulibacter* performed a PAO metabolism with a P/VFA ratio of 0.64 P-mol/C-mol; in contrast, type I members displayed a mixed PAO-GAO metabolism with a P/VFA ratio of 0.22 P-mol/C-mol and a correspondingly high PHV content in the PHA. Although a majority of the detectable *Ca. Accumulibacter* OTUs in the three plants seemed to be type II members, the predominance of different species in different plants might also have contributed to differences in the stoichiometric values. Additionally, the conversion of VFAs into PHA requires reducing equivalents, where for *Ca. Accumulibacter*, these reducing equivalents can be obtained from glycolysis and/or the anaerobic operation of the reductive branch of the TCA cycle (Comeau et al., 1986; Mino et al., 1987; Schuler and Jenkins, 2003; Hesselmann et al., 2000; Pijuan et al., 2008; Zhou et al., 2010). The involvement of the TCA cycle results in a higher PHV content. The contribution of each pathway varies among different *Ca. Accumulibacter* taxa (Majed et al., 2012), and depends on the availability of glycogen in the cells (Zhou et al., 2009). PAOs in full-scale temperate EBPR plants often employ the anaerobic TCA cycle in addition to, or instead of, the glycolysis pathway (Lanham et al., 2013). Law et al. (2016) also suggested that glycolysis and the TCA cycle were of equal importance in supplying reducing power in a full-scale tropical EBPR system, based on both stoichiometric and metatranscriptomic results, where genes in both pathways were highly expressed at approximately the same levels. The relatively lower anaerobic Gly/VFA and aerobic Gly/PHA values observed in

WWTP1, together with PHV/PHB values comparable to those found in WWTP3 (with a higher relative abundance of GAOs), would suggest a greater use of the TCA cycle in WWTP1.

In general, based on the PHA/VFA ratios, most of the PHA was composed of PHB when acetate was supplied. Anaerobic Gly/VFA and aerobic Gly/PHA ratios were also at a low level. Additionally, the P/VFA ratios were close to the model values of *Ca. Accumulibacter*, suggesting that *Ca. Accumulibacter* (and probably other PAOs) could effectively acquire organic carbon in the presence of high numbers of GAOs. This was particularly evident in WWTP3, where the relative abundance of *Ca. Competibacter* (2.40-4.35%) and *Defluviicoccus* (3.04-3.43%) was 5.4 to 6.7 times higher than that of *Ca. Accumulibacter* (0.95-1.40%) (Fig. 2B). The stoichiometric values were not different from those observed at other plants, implying that these GAOs were not significantly affecting carbon uptake and P removal by PAOs.

When primary effluent was used, the observed P/VFA values were much higher compared to acetate or propionate, suggesting that other organic compounds present in the wastewater were utilised by PAOs and contributed to additional P-release (Supplementary Fig. S3C). Higher Gly/VFA ratios (compared to those when acetate or propionate were supplied) were also observed, together with elevated PHA/VFA values as well as a high PHV content of the PHA, suggesting that both PAOs and GAOs were more active. Overall it appears, in view of the high observed P/VFA ratios, that both PAOs and GAOs could access other organic compounds from the wastewater. More work is needed to understand the roles of carbon sources in mediating the interactions between PAOs and GAOs in full-scale EBPR systems.

Conclusions

- Three WWTPs in tropical Singapore showed high in-situ EBPR activities. Each plant possessed a characteristic microbial community composed of diverse putative PAOs with multiple OTUs (affiliated to *Ca. Accumulibacter*, *Tetrasphaera*, *Dechloromonas* and *Ca. Obscuribacter*), highlighting that PAOs commonly found in temperate EBPR systems also thrive at higher temperatures.
- Despite differences in composition, all microbial communities were capable of P-release by using a wide range of carbohydrates, lipids, and protein hydrolysates. This flexibility is important in dynamic environments and with complex organic-carbon mixtures, as found in full-scale WWTPs.
- The PAO community composition was highly correlated with the EBPR-associated carbon usage profiles, attributable in part to the different capabilities of specific taxa in using specific carbon sources.
- *Ca. Accumulibacter* was the most dominant group of PAOs in the WWTPs, and VFAs were the most effective carbon sources for release and uptake of P. These findings highlight the important role of *Ca. Accumulibacter* and VFAs, similar to temperate EBPR systems.
- Even within the lineage of *Ca. Accumulibacter*, different taxa were associated with the P-release induced by distinct carbon sources, implying that *Ca. Accumulibacter* contains diverse members with versatile carbon metabolisms. In complex and variable environments, these differences may allow them to occupy different ecological niches, where they can co-exist and contribute to a robust EBPR.
- Apart from *Ca. Accumulibacter* and *Tetrasphaera*, other PAOs contributed to the EBPR, However, currently, very limited information is available on those PAOs and future work should investigate their roles and metabolisms in full-scale plants.

Acknowledgements

This research was supported by the Singapore National Research Foundation and the Ministry of Education under the Research Centre of Excellence Programme, and by a research grant from the National Research Foundation under its Environment and Water Industry Programme (project number 1102-IRIS-10-02), administered by PUB-Singapore's national water agency. We thank Mr. Larry Liew and staff from PUB, Singapore's National Water Agency for the assistance in sample collection. Dr. Guanglei Qiu acknowledges the support of National Natural Science Foundation of China (No. 51808297).

References

- Abu-Gharach, Z.H., Randall, C.W., 1991. The effect of organic compounds on biological phosphorus removal. *Water Sci. Technol.* 23, 585–594.
- Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R. C., May, J. W., G., Vasiliadis., Seviour, R.J., 2007. Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor. *Appl. Environ. Microbiol.* 73, 2257–2270.
- Albertsen, M., Hansen, L.B.S., Saunders, A.M. Nielsen P.H., Nielsen, K.L., 2012. A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal. *ISME J.* 6, 1094–1106.
- Albertsen, M., Karst, S.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H., 2015. Back to basics—the influence of DNA extraction and primer choice on phylogenetic analysis in activated sludge communities. *PLoS ONE* 10:e0132783.
- APHA, 1999. *Standard Methods for the Examination of Water and Wastewater*, nineteenth ed. American Public Health Association. Inc., Washington DC.

- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57 (1): 289–300.
- Burow, L., Mabbett, A., McEwan, A., Bond, P., Blackall, L., 2008. Bioenergetic models for acetate and phosphate transport in bacteria important in enhanced biological phosphorus removal. *Environ. Microbiol.* 10: 87–98.
- Comeau, Y., Hall, K.J., Hancock, R.E.W., Oldham, W. K. 1986. Biochemical model for enhanced biological phosphorus removal. *Water Res.* 20, 1511–1521.
- Cokro, A.A., Law, Y., Williams, R.B.H., Cao, Y., Nielsen, P.H., Wuertz, S., 2017. Non-denitrifying polyphosphate accumulating organisms obviate requirement for anaerobic condition. *Water Res.* 111, 393–403.
- Chua, A.S.M., Onuki, M., Satoh, H., Mino, T., 2006. Examining substrate uptake patterns of *Rhodocyclus*-related PAO in full-scale EBPR plants by using the MARFISH technique. *Wat. Sci. Technol.* 54(1), 63–70.
- Crocetti, G. R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA directed probes for their detection and quantitation. *Environ. Microbiol.* 66, 1175–1182.
- Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M., The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* 22, 434–444.
- Freitas, F., Temudo, M.F., Carvalho, G., Oehmen, A., Reis, M.A.M., 2009. Robustness of sludge enriched with short SBR cycles for biological nutrient removal. *Bioresour. Technol.* 100, 1969–1976.

- Flowers, J.J., He, S., Malfatti, S., del Rio, T.G., Tringe, S.G., Hugenholtz, P., McMahon, K.D., 2013. Comparative genomics of two '*Candidatus Accumulibacter*' clades performing biological phosphorus removal. *ISME J.* 7, 2301–2314.
- Flowers, J.J., He, S., Yilmaz, S., Noguera, D.R., McMahon, K.D., 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different "*Candidatus Accumulibacter*" clades. *Environ. Microbiol. Rep.* 1(6), 583–588.
- Fukushima, T., Uda, N., Okamoto, M., Onuku, M., Satoh, H., Mino, T., 2007. Abundance of *Candidatus 'Accumulibacter phosphatis'* in enhanced biological phosphorus removal activated sludge acclimatized with different carbon sources. *Microbes Environ.* 22 (4), 346–354.
- Ge, H., Batstone, D.J., Keller, J., 2015. Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade Comamonadaceae. *Water Res.* 69, 173–182.
- Gu, A.Z., Saunders, A.M., Neethling, J.B., Stensel, H.D., Blackall, L.L., 2008. Functionally relevant microorganisms to enhanced biological phosphorus removal performance at full-scale wastewater treatment plants in the United States. *Water Environ. Res.* 80 (8), 688–698.
- Günther, S., Trutnau, M., Kleinstaub, S., Hause, G., Bley, T., Röske, I., Harms, H., Müller, S., 2009. Dynamics of polyphosphate accumulating bacteria in wastewater treatment plant microbial communities detected via DAPI (4',6'-diamidino-2-phenylindole) and tetracycline labelling. *Appl. Environ. Microbiol.* 75, 2111–2121.
- Hanada, S., Liu, W.T., Shintani, T., Kamagata, Y., Nakamura, K., 2002. *Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* 52, 883–887.

- 632 He, S., McMahon K.D., 2011. Microbiology of 'Candidatus Accumulibacter' in activated sludge.
633 Microb. Biotechnol. 4(5), 603–619.
- 634 He, S., Gall, D.L., McMahon, K.D., 2007. 'Candidatus Accumulibacter' population structure in
635 enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes.
636 Appl. Environ. Microbiol. 73, 5865–5874.
- 637 He, S., Gu, A.Z., McMahon, K.D., 2008. Progress toward understanding the distribution of
638 *Accumulibacter* among full-scale enhanced biological phosphorus removal systems. Microb.
639 Ecol. 55, 229–236.
- 640 Hesselmann, R.P.X., Von Rummell, R., Resnick, S.M., Hany, R., Zehnder, A.J.B., 2000. Anaerobic
641 metabolism of bacteria performing enhanced biological phosphate removal. Water Res. 34,
642 3487–3494.
- 643 Kawakoshi, A., Nakazawa, H., Fukada, J., Sasagawa, M., Katano, Y., Nakamura, S., Hosoyama, A.,
644 Natsuko H.S., Hanada, I.S., 2012. Deciphering the genome of polyphosphate accumulating
645 actinobacterium *Microthrix phosphovorans*. DNA Res. 19, 383–394.
- 646 Kong, Y.H., Nielsen, J.L., Nielsen, P.H., 2004. Microautoradiographic study of Rhodocyclus-related
647 polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal
648 plants. Appl. Environ. Microbiol. 70(9), 5383–5390.
- 649 Kong, Y.H., Nielsen, J.L., Nielsen, P.H., 2005. Identity and ecophysiology of uncultured
650 Actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological
651 phosphorus removal plants. Appl. Environ. Microbiol. 71(7), 4076–4085.
- 652 Kong, Y. H., Xia, Y., Nielsen, J.L., Nielsen, P.H., 2007. Structure and function of the microbial
653 community in a full-scale enhanced biological phosphorus removal plant. Microbiology, 153,
654 4061–4073.

- Kristiansen, R., Nguyen, H.T.T., Saunders, A.M., Nielsen, J.L., Wimmer, R., Le, V.Q., McIlroy, S.J., Petrovski, S., Seviour, R.J., Calteau, A., Nielsen, K. L., Nielsen, P.H., 2013. A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal. ISME J. 7, 543–554.
- Lanham, A.B., Oehmen, A., Saunders, A.M., Carvalho, G., Nielsen, P.H., Reis M.A.M., 2013. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. Water Res 47, 7032–7041.
- Law, Y., Kirkegaard, R.H., Cokro, A.A., Liu, X., Arumugam, K., Xie, C., Stokholm-Bjerregaard, M., Drautz-Moses, D.I., Nielsen, P.H., Wuertz, S., Williams, R.B.H., 2016. Integrative microbial community analysis reveals full-scale enhanced biological phosphorus removal under tropical conditions. Sci. Rep. 6, 25719.
- Lopez-Vazquez, C.M., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., van Loosdrecht, M.C.M., 2008. Factors affecting the microbial populations at full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plants in The Netherlands. Water Res. 42, 2349–2360.
- Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Res. 43(2), 450–462.
- Lu, H., Oehmen, A., Viridis, B., Keller, J., Yuan, Z., 2006. Obtaining highly enriched cultures of *Candidatus Accumulibacter phosphatis* through alternating carbon sources. Water Res. 40 (20), 3838–3848.
- Majed, N., Chernenko, T., Diem, M., Gu, A.Z., 2012. Identification of functionally relevant populations in enhanced biological phosphorus removal processes based on intracellular polymers profiles and insights into the metabolic diversity and heterogeneity. Environ. Sci. Technol. 46, 5010–5017.

- 679 Mao, Y., Graham, D. W., Tamaki, H., Zhang, T., 2015. Dominant and novel clades of *Candidatus*
 680 *Accumulibacter phosphatis* in 18 globally distributed full-scale wastewater treatment plants. *Sci.*
 681 *Rep.* 5, 11857.
- 682 Marques, R., Santos, J., Nguyen, H., Carvalho, G., Noronha, J.P., Nielsen, P.H., Reis, M.A.M.,
 683 Oehmen, A., 2017. Metabolism and ecological niche of *Tetrasphaera* and *Ca. Accumulibacter* in
 684 enhanced biological phosphorus removal. *Water Res.* 122, 159–171.
- 685 McIlroy, S.J., Nittami, T., Seviour, E.M., Seviour, R.J., 2010. Filamentous members of cluster III
 686 *Defluviicoccus* have the in situ phenotype expected of a glycogen-accumulating organism in
 687 activated sludge. *FEMS Microbiol. Ecol.* 74(1), 248–256.
- 688 McIlroy, S. J., Saunders, A. M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A. A., Karst,
 689 S.M., Nielsen, J.L., Nielsen P.H., 2015. MiDAS: the field guide to the microbes of activated
 690 sludge. *Database* 2015, 1–8.
- 691 McIlroy, S.J., Starnawska, A., Starnawski, P., Saunders, A.M., Nierychlo, M., Nielsen, P.H., Nielsen,
 692 J.L., 2016. Identification of active denitrifiers in full-scale nutrient removal wastewater
 693 treatment systems. *Environ. Microbiol.* 18, 50–64.
- 694 Mielczarek, A.T., Nguyen, H.T.T., Nielsen, J.L., Nielsen, P.H., 2013. Population dynamics of
 695 bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment
 696 plants. *Water Res.* 47, 1529–1544.
- 697 Mino, T., Loosdrecht, M., Heijnen, J.J., 1998. Microbiology and biochemistry of the enhanced
 698 biological phosphate removal process. *Water Res.* 32 (11), 3193-3207.
- 699 Muszyński, A., Załęska-Radziwiłł, M., 2015. Polyphosphate Accumulating organisms in treatment
 700 plants with different wastewater composition. *Archit. Civil Eng. Environ.* 8, 99–105. Nakamura,
 701 K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Masuda, K., and Kamagata, Y., 1995.

- 702 *Microtholunatus phosphovor* gen. nov., sp. nov., a new Gram-positive polyphosphate-
 703 accumulating bacterium isolated from activated sludge. Int. J. Syst. Bacteriol. 45, 17–22.
- 704 Nguyen, H.T.T., Kristiansen, R., Vestergaard, M., Wimmer, R., Nielsen, P.H., 2015. Intracellular
 705 accumulation of glycine in polyphosphate-accumulating organisms in activated sludge, a novel
 706 storage mechanism under dynamic anaerobic-aerobic conditions. Appl. Environ. Microbiol.
 707 81(14), 4809–4818.
- 708 Nguyen, H.T.T., Le, V.Q., Hansen, A.A., Nielsen, J.L., Nielsen, P.H., 2011. High diversity and
 709 abundance of putative polyphosphate accumulating *Tetrasphaera*-related bacteria in activated
 710 sludge systems. FEMS Microbiol. Ecol. 76, 256–267.
- 711 Nguyen, H.T.T., Nielsen, J.L., Nielsen, P.H., 2012. “*Candidatus Halomonas phosphatis*”, a novel
 712 polyphosphate-accumulating organism in full-scale enhanced biological phosphorus removal
 713 plants. Environ. Microbiol. 14, 2826–2837.
- 714 Nielsen, P.H. Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y., Hansen,
 715 A.A., Vollertsen, J., 2010. A conceptual ecosystem model of microbial communities in
 716 enhanced biological phosphorus removal plants. Water Res. 44, 5070–5088.
- 717 Oehmen, A., Keller-Lehmann, B., Zeng, R.J., Yuan, Z.G., Keller, J., 2005a. Optimisation of poly-
 718 beta-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus
 719 removal systems. J. Chromatogr. A 1070, 131–136.
- 720 Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2005b. Comparison of acetate and propionate
 721 uptake by polyphosphate accumulating organisms and glycogen accumulating organisms.
 722 Biotechnol. Bioeng. 91(2), 162–168.

- Oehmen, A., Zeng, R.J., Saunders, A.M., Blackall, L.L., Keller J., Yuan Z., 2006. Anaerobic and aerobic metabolism of glycogen accumulating organisms selected with propionate as the sole carbon source. *Microbiology*, 152, 2767–2778.
- Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale, *Water Res.* 41, 2271–2300.
- Ong, Y.H., Chua, A.S.M., Fukushima, T., Ngoh, G.C., Shoji, T., Michinaka, A., 2014. High-temperature EBPR process: the performance, analysis of PAOs and GAOs and the fine-scale population study of *Candidatus* “*Accumulibacter phosphatis*”. *Water Res.* 64, 102–112.
- Oyserman, B.O., Noguera, D.R., del Rio, T.G., Tringe, S.G., McMahon K.D., 2016. Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus* *Accumulibacter phosphatis*. *ISME J*, 10, 810–822.
- Panswad, T., Doungchai, A., Anotai, J. 2003. Temperature effect on microbial community of enhanced biological phosphorus removal system. *Water Res.* 37, 409–415.
- Pijuan, M. Oehmen, A., Baeza, J.A., Casas, C., Yuan, Z., 2008. Characterizing the biochemical activity of full-scale enhanced biological phosphorus removal systems: A comparison with metabolic models. *Biotech. Bioeng.* 99, 170–179.
- Rubio-Rincón, F.J., Welles, L., Lopez-Vazquez, C.M., Nierychlo, M., Abbas, B., Geleijnse, M., Nielsen, P.H., van Loosdrecht, M.C.M., Brdjanovic, D. 2017. Long-term effects of sulphide on the enhanced biological removal of phosphorus: The symbiotic role of *Thiothrix caldifontis*. *Water Res.* 116, 53–64.
- Saunders, A.M., Albertsen, M., Vollertsen, J., Nielsen, P.H., 2016. The activated sludge ecosystem contains a core community of abundant organisms. *ISME J.* 10, 11–20.

- Seviour, R.J., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol. Rev.* 27, 99–127.
- Schuler, J., Jenkins, D., 2003. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents. Part III: Anaerobic sources of reducing power. *Water Environ. Res.* 75(6), 512–522.
- Shen, N., Chen, Y., Zhou, Y., 2017. Multi-cycle operation of enhanced biological phosphorus removal (EBPR) with different carbon sources under high temperature *Water Res.* 114, 308–315.
- Shen, N., Zhou, Y., 2016. Enhanced biological phosphorus removal with different carbon sources. *Appl. Microbiol. Biotechnol.* 100, 4735–4745.
- Silva, A.F., Carvalho, G., Oehmen, A., 2012. Microbial population analysis of nutrient removal-related organisms in membrane bioreactors. *Appl. Microbiol. Biotechnol.* 93, 2171–2180.
- Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1995. A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. *Biotechnol. Bioeng.* 47(3), 277–287.
- Soo, R.M., Skennerton, C.T., Sekiguchi, Y., Imelfort, M., Paech, S. J., Dennis, P.G., Steen, J.A., Parks, D.H., Tyson, G.W., Hugenholtz, P., 2014. An expanded genomic representation of the phylum Cyanobacteria. *Genome Biol. Evol.* 6, 1031–1045.
- Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. *Front. Microbiol.* 8: 718.

- 767 Tu, Y., Schuler, A.J., 2013. Low acetate concentrations favor polyphosphate-accumulating
768 organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal
769 from wastewater. *Environ. Sci. Technol.* 47, 3816–3824.
- 770 Ubakata, Y., Takii, S., 1998. Some physiological characteristics of phosphate removing bacterium,
771 *Microthrix phosphovorans*, and a simplified isolation and identification method for phosphate-
772 removing bacteria. *Water Sci. Technol.* 38, 149–157.
- 773 Welles, L., Tian, W.D., Saad, S., Abbas, B., Lopez-Vazquez, C.M., Hooijmans, C.M., van
774 Loosdrecht, M.C.M., Brdjanovic, D., 2015. Accumulibacter clades Type I and II performing
775 kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate
776 uptake. *Water Res.* 83, 354–366.
- 777 Whang, L.M., Park, J.K., 2002. Competition between polyphosphate- and glycogen-accumulating
778 organisms in biological phosphorus removal systems-effect of temperature. *Water Sci. Technol.*
779 46 (1-2), 191–194.
- 780 Winkler, M.K.H., Bassin, J.P., Kleerebezem, R., de Bruin, L.M.M., van den Brand, T.P.H., van
781 Loosdrecht, M.C.M., 2011. Selective sludge removal in a segregated aerobic granular biomass
782 system as a strategy to control PAO-GAO competition at high temperature. *Water Res.* 45,
783 3291-3299.
- 784 Yuan, Z., Pratt, S., Batstone, D.J., 2012. Phosphorus recovery from wastewater through microbial
785 processes. *Curr. Opin. Biotechnol.* 23 (6), 878–883.
- 786 Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z., Keller, J., 2003. Metabolic model for glycogen-
787 accumulating organisms in anaerobic/aerobic activated sludge systems. *Biotech. Bioeng.* 81(1),
788 92–105.

- 789 Zengin, G.E., Artan, N., Orhon, D., Satoh, H., Mino, T., 2011. Effect of aspartate and glutamate on
790 the fate of enhanced biological phosphorus removal process and microbial community structure.
791 Bioresour. Technol 102(2), 894–903.
- 792 Zhang, A.N., Mao, Y., Zhang, T., 2017. Development of quantitative real-time PCR assays for
793 different clades of “*Candidatus Accumulibacter*”. Sci. Rep. 6, 23993.
- 794 Zhang, Z., Li, H., Zhu, J., Liu, W., Xu, X. 2011. Improvement strategy on enhanced biological
795 phosphorus removal for municipal wastewater treatment plants: Full-scale operating parameters,
796 sludge activities, and microbial features. Bioresour. Technol. 102, 4646–4653.
- 797 Zhou, Y., Pijuan, M., Zeng, R.J., Yuan, Z., 2009. Involvement of the TCA cycle in the anaerobic
798 metabolism of polyphosphate accumulating organisms (PAOs). Water Res. 43 (5), 1330–1340.
- 799 Zhou, Y., Pijuan, M., Oehmen, A., Yuan, Z., 2010. The source of reducing power in the anaerobic
800 metabolism of polyphosphate accumulating organisms (PAOs) – a mini-review. Water Sci.
801 Technol. 61 (7) 1653-1662.

1 **Table 1** Operating conditions, primary effluent and secondary effluent characteristics and EBPR activity at the three WWTPs in Singapore

Characteristic	Parameter, unit	WWTP1	WWTP2	WWTP3
Operating conditions	Process configuration	5-stage step feeding	Modified Ludzack–Ettinger (MLE)	Anoxic/Anaerobic/Aerobic-MBR
	Treatment capacity, m ³ /day	361,000	205,000	68,000
	SRT, day	5.0	5.0	15 - 20
	HRT, h	5.6	6.8	10 - 12
	T, °C	28.7 - 31.2	29.8 - 31.1	29.3 - 31.6
	pH	6.32 - 7.49	6.82 - 7.67	6.62 - 8.70
	MLSS ^a , g/L	2.30 - 2.76	2.18 - 2.45	3.33 - 3.80
	MLVSS ^a , g/L	1.70 - 1.87	1.84 - 1.95	2.27 - 2.56
Primary effluent	TCOD, mg/L	237 - 324	337 - 381	348 - 394
	PO ₄ ³⁻ P, mg/L	4.44 - 5.31	5.14 - 7.38	2.46 - 5.39
	TP, mg/L	5.53 - 6.52	6.02 - 8.19	3.48 - 6.37
	NH ₄ ⁺ -N, mg/L	38.7 - 42.8	37.7 - 43.8	36.2 - 43.9
	TN, mg/L	42.6 - 50.1	40.9 - 44.7	38.1 - 45.6

	TOC, mg/L	37.8 - 62.2	63.8 - 69.9	68.6 - 87.1
	Acetate, mg/L	26.9 - 31.5	50.3 - 62.5	32.7 - 44.5
	Propionate, mg/L	3.60 - 4.41	4.90 - 8.37	5.41 - 7.20
Secondary effluent	TCOD, mg/L	10 - 12	15 - 20	13 - 21
	PO ₄ ³⁻ P, mg/L	0.51 - 1.61	0	0 - 2.38
	TP, mg/L	0.62 - 1.70	0	0 - 2.29
	NH ₄ ⁺ -N, mg/L	0.57 - 3.93	0 - 12.8	0-0.56
	NO ₃ ⁻ -N, mg/L	2.11 - 3.19	6.18-9.61	6.47 - 9.28
	NO ₂ ⁻ -N, mg/L	0.24 - 0.34	0-1.78	0
	TN, mg/L	3.69 - 8.76	10.6 - 21.7	8.89 - 11.4
	TOC, mg/L	5.17 - 6.31	6.75 - 9.91	8.38 - 10.6
In situ EBPR activity	P-release ^b , mgP/mgVSS	5.16 - 5.58	6.79 - 16.7	3.36 - 5.26
	P-uptake ^c , mgP/mgVSS	6.21 - 6.50	9.19 - 18.2	3.63 - 6.35

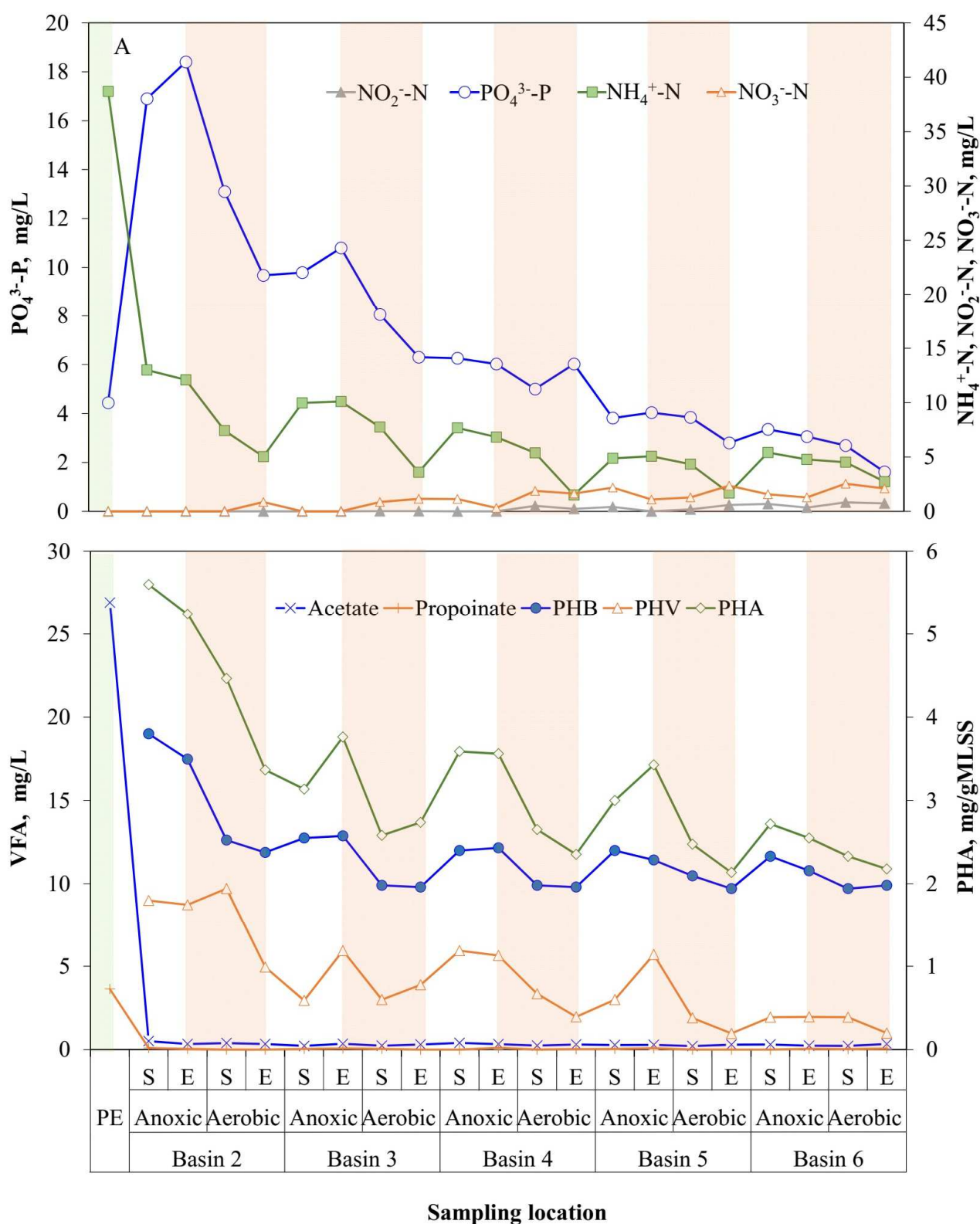
^a The values show the concentrations at the end of the aerobic stage; ^b Calculated by subtracting the primary effluent PO₄³⁻-P concentration from the PO₄³⁻-P concentration measured at the end of the anaerobic/anoxic stage; ^c Calculated as difference between the PO₄³⁻-P concentration at the end of the anaerobic/anoxic stage and that at the end of the aerobic stage.

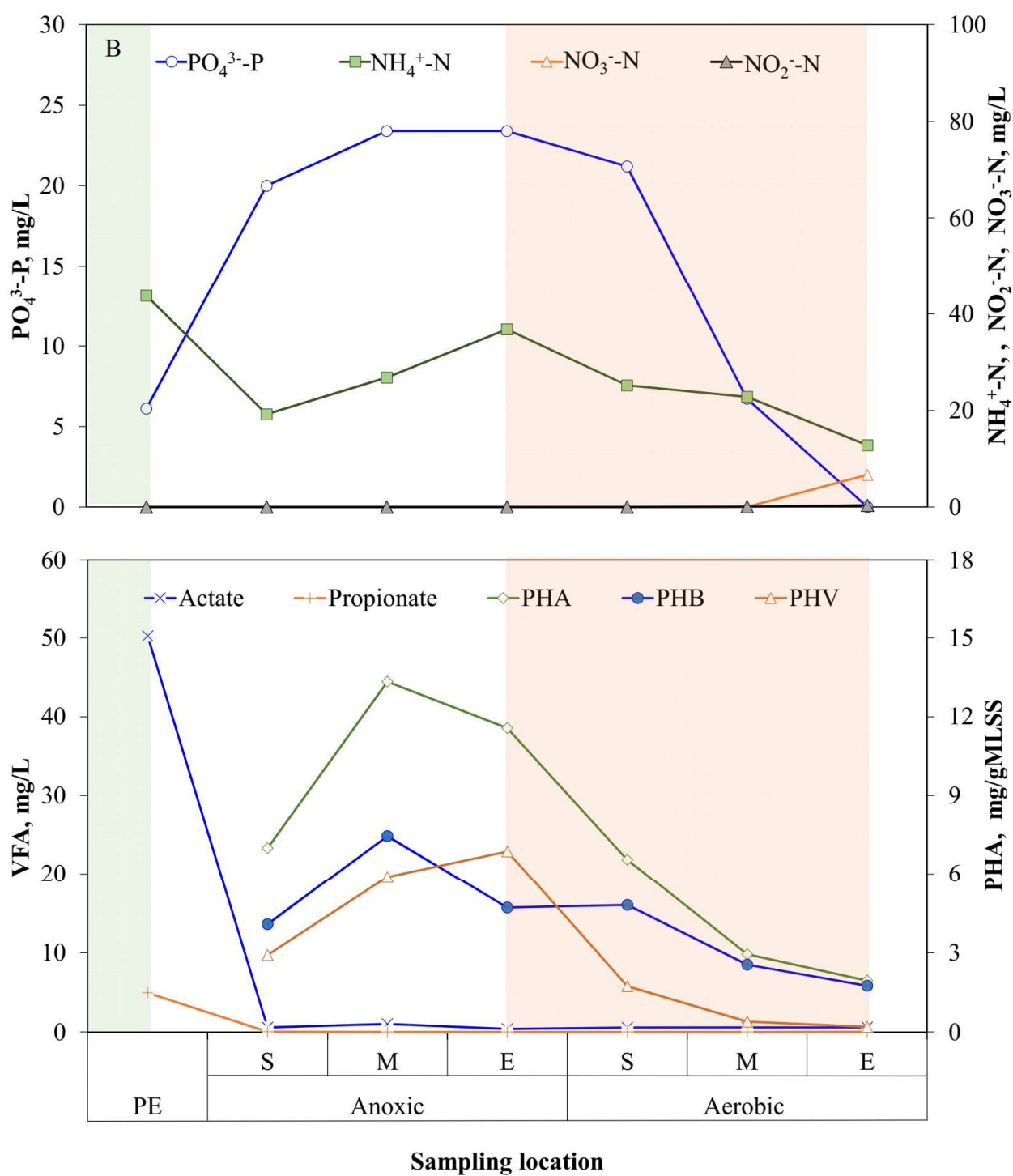
Table 2 Stoichiometric ratios of phosphorus and carbon transformations during the anaerobic-aerobic cycle tests. Values represent the mean with standard deviation in parentheses (n = 3).

Carbon source	Origin	Anaerobic stoichiometry						Aerobic stoichiometry		Reference
		P/VFA ^a	PHA/VFA ^b	PHB/VFA ^c	Gly/VFA ^f	PHV/VFA ^d	PHV/PHB ^e	P/PHA ^h	Gly/PHA ⁱ	
		P-mol/C-mol	C-mol/C-mol					P-mol/C-mol	C-mol/C-mol	
Acetate	WWTP1	0.35 (0.01)	0.63 (0.06)	0.52 (0.09)	0.58 (0.10)	0.11 (0.02)	0.20 (0.08)	0.65 (0.22)	0.22 (0.02)	This study
	WWTP2	0.66 (0.06)	0.78 (0.24)	0.69 (0.18)	0.61 (0.26)	0.09 (0.07)	0.11 (0.07)	1.21 (0.46)	0.17 (0.05)	This study
	WWTP3	0.41 (0.08)	0.78 (0.10)	0.65 (0.04)	0.64 (0.15)	0.13 (0.07)	0.20 (0.10)	0.57 (0.08)	0.26 (0.01)	This study
	PAO TCA model	0.50	0.90	0.90	0.00	0.00	0.00	-	-	Comeau et al., 1986
	PAO Gly model	0.50	1.33	1.33	0.50	0.00	0.00	0.41	0.42	Smolder et al., 1995
	PAO TCA/Gly model	0.37	1.40	1.11	0.60	0.29	0.26	-	-	Hesselmann et al., 2000
	<i>Ca. Accumulibacter</i> Type I	0.64	1.36	1.27	0.29	0.09	0.07	-	-	Welles et al., 2015
	<i>Ca. Accumulibacter</i> Type II	0.22	1.47	1.24	0.96	0.23	0.19	-	-	Welles et al., 2015
	Full-scale sludge	0.30 - 1.30	0.67 - 1.74	- ^g	0.04 - 0.82	-	-	0.3-1.8	0.2-1.1	Lanham et al., 2013
	Full-scale sludge	0.33 - 0.45	-	-	-	-	-	-	-	Lopez-Vazquez et al., 2008
	Full-scale sludge	0.29 - 0.75	-	-	-	-	-	-	-	Gu et al., 2008
	Full-scale sludge	0.42 - 0.59	1.20 - 1.35	1.05 - 1.08	0.34 - 0.40	0.15 - 0.27	0.14 - 0.25	-	-	Pijuan et al., 2008

	GAO Model	0.00	1.86	1.36	1.12	0.46	0.34	0.00	0.65	Zeng et al., 2003
Propionate	WWTP1	0.38 (0.04)	0.56 (0.10)	0.04 (0.02)	0.69 (0.25)	0.53 (0.07)	-	0.49 (0.37)	0.35 (0.14)	This study
	WWTP2	0.60 (0.07)	0.60 (0.13)	0.05 (0.04)	0.68 (0.19)	0.48 (0.08)	-	1.06 (0.31)	0.52 (0.15)	This study
	WWTP3	0.46 (0.16)	0.61 (0.16)	0.06 (0.04)	0.71 (0.22)	0.55 (0.12)	-	0.31 (0.12)	0.38 (0.16)	This study
	PAO model	0.42	1.22	0.00	0.33	0.56	-	-	-	Oehmen et al., 2005b
	GAO model (<i>Deftluviicoccus</i>)	0.00	1.50	0.00	0.67	0.83	-	-	-	Oehmen et al., 2006
Primary effluent	WWTP1	0.63 (0.03)	1.39 (0.00)	0.72 (0.06)	1.15 (0.05)	0.59 (0.08)	0.83 (0.19)	0.90 (0.28)	0.38 (0.13)	This study
	WWTP2	1.00 (0.05)	1.20 (0.16)	0.64 (0.03)	1.74 (0.42)	0.55 (0.13)	0.86 (0.11)	0.78 (0.28)	0.18 (0.11)	This study
	WWTP3	0.94 (0.00)	1.51 (0.08)	0.90 (0.07)	1.49 (0.16)	0.57 (0.05)	0.63 (0.14)	0.71 (0.07)	0.30 (0.18)	This study

^aP-release to VFA-uptake molar ratio; ^bPHA-formation to VFA-uptake molar ratio; ^cPHB-formation to VFA-uptake molar ratio; ^dPHV-formation to VFA-uptake molar ratio; ^ePHB to PHV molar ratio in the PHA; ^fGlycogen-consumption to VFA-uptake molar ratio; ^gvalues not available; ^hP-uptake to PHA-consumption molar ratio; ⁱGlycogen-formation to PHA-consumption molar ratio.





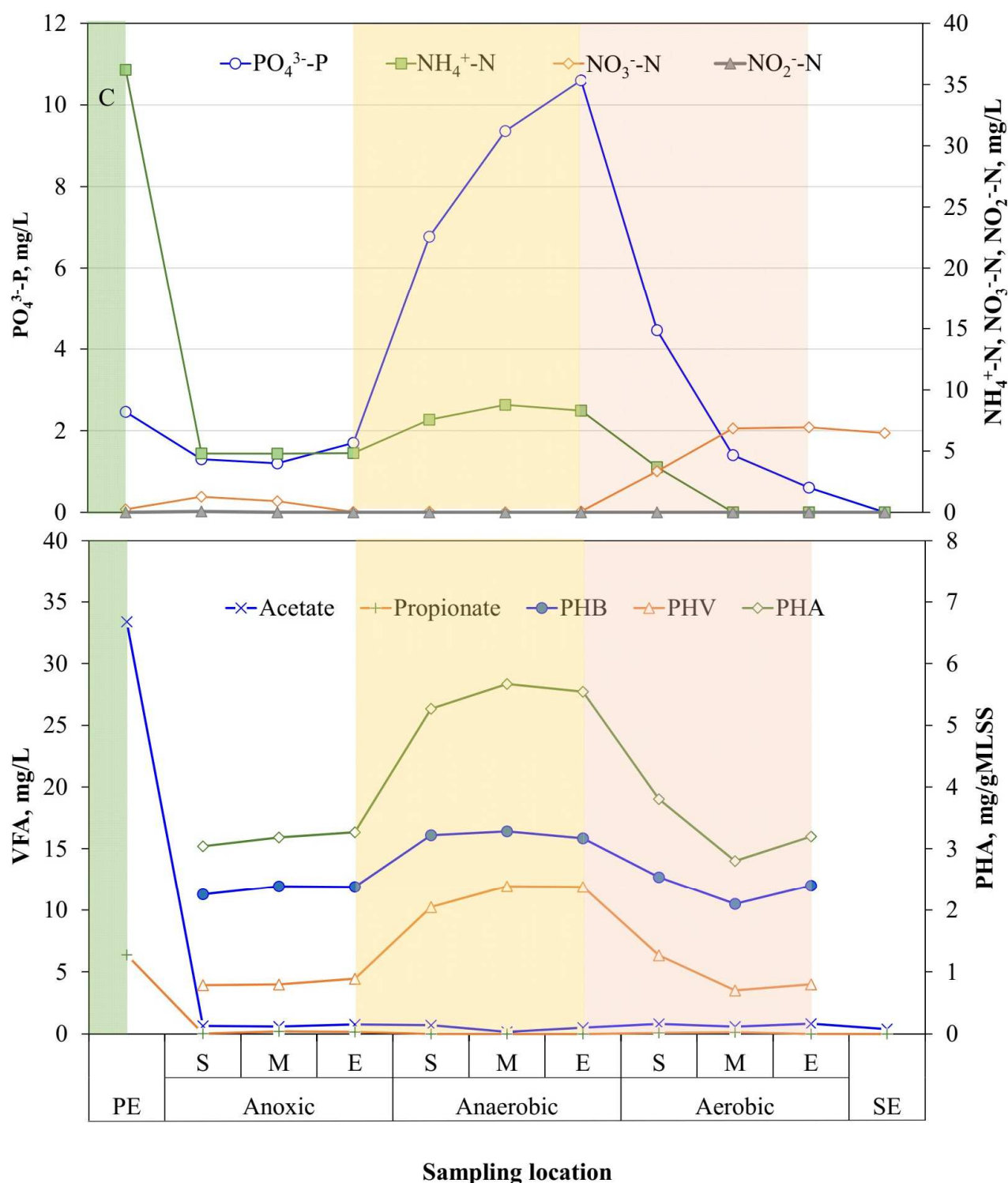
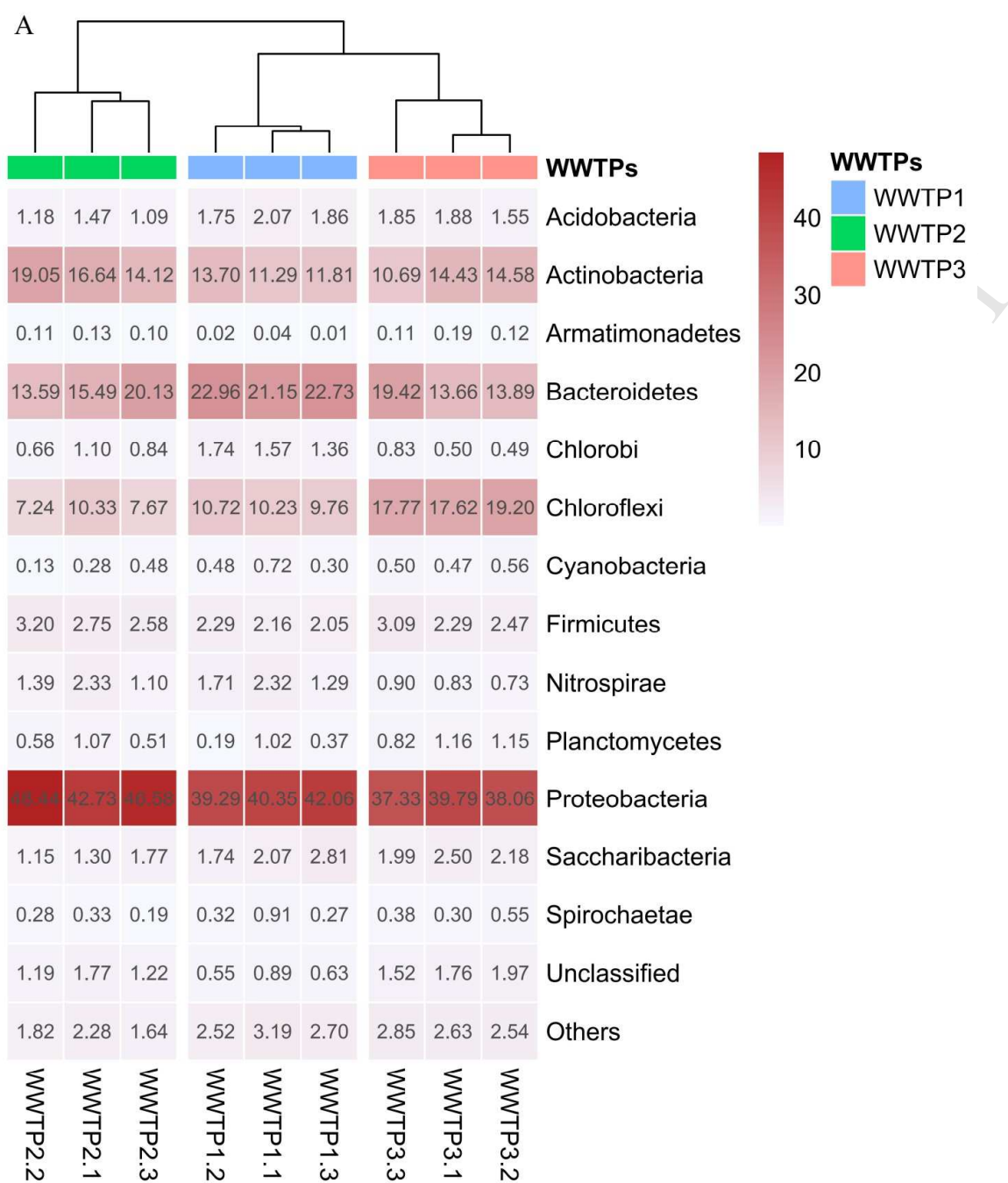
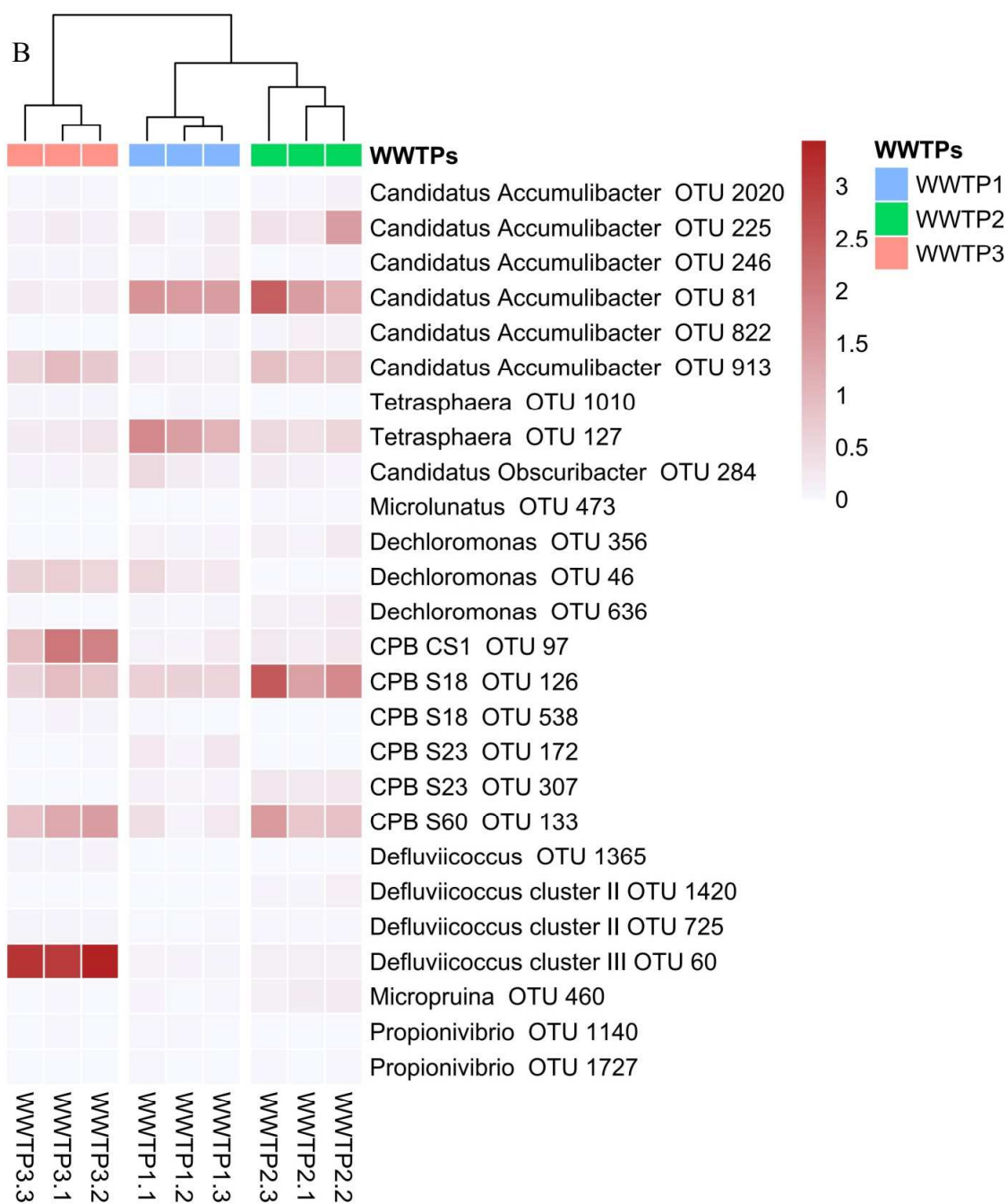


Figure 1 - Nutrient concentrations at the three WWTPs plants showing EBPR activity. (A), WWTP1; (B), WWTP2; (C), WWTP3. PE, primary effluent; S, start; M, mid; E, end; SE, secondary effluent. Green shading, PE; yellow shading, anaerobic stage; orange shading, aerobic stage.





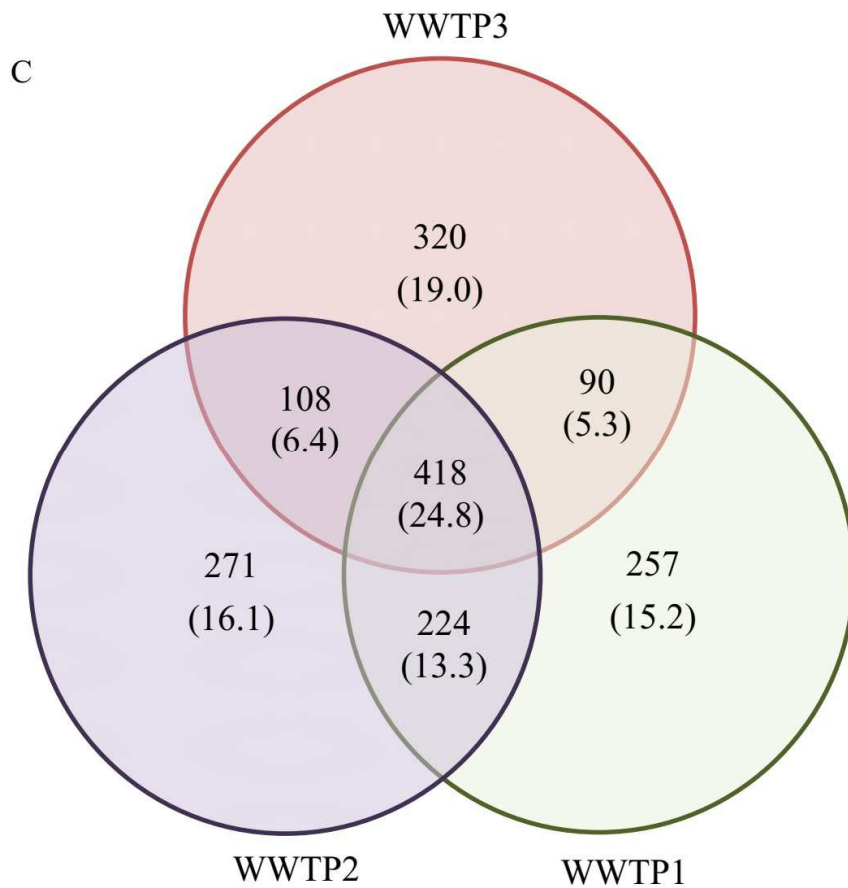


Figure 2 - Bacterial community composition at three WWTPs in Singapore. (A), communities at phylum level, (B), putative PAO and GAO community communities (only OTUs with relative abundance >0.02% are included); and (C) Venn diagram of the shared OTUs (with no less than 3 reads) among different WWTPs as suggested by 16S rRNA gene amplicon analysis. Parentheses show percentages.

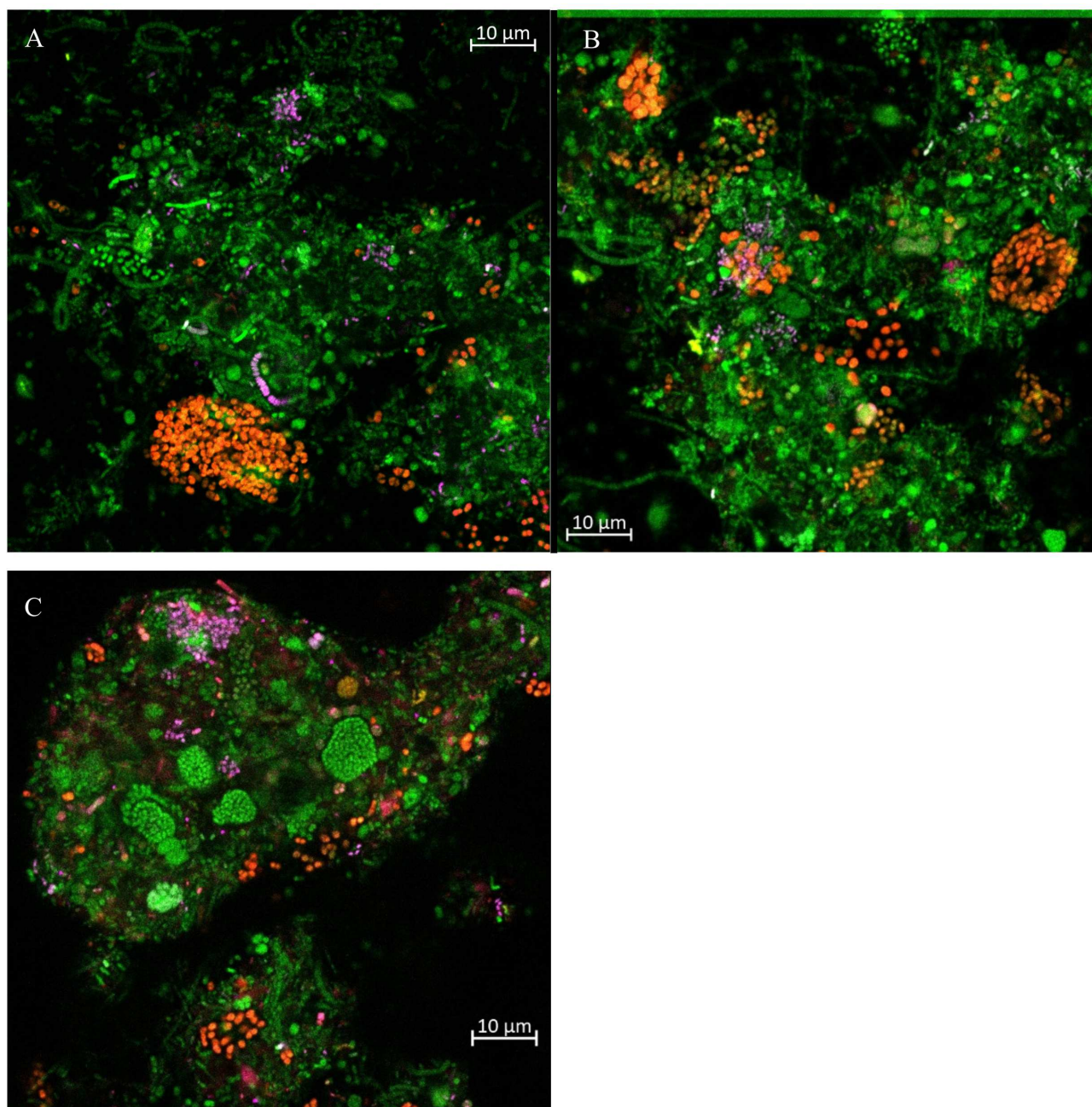
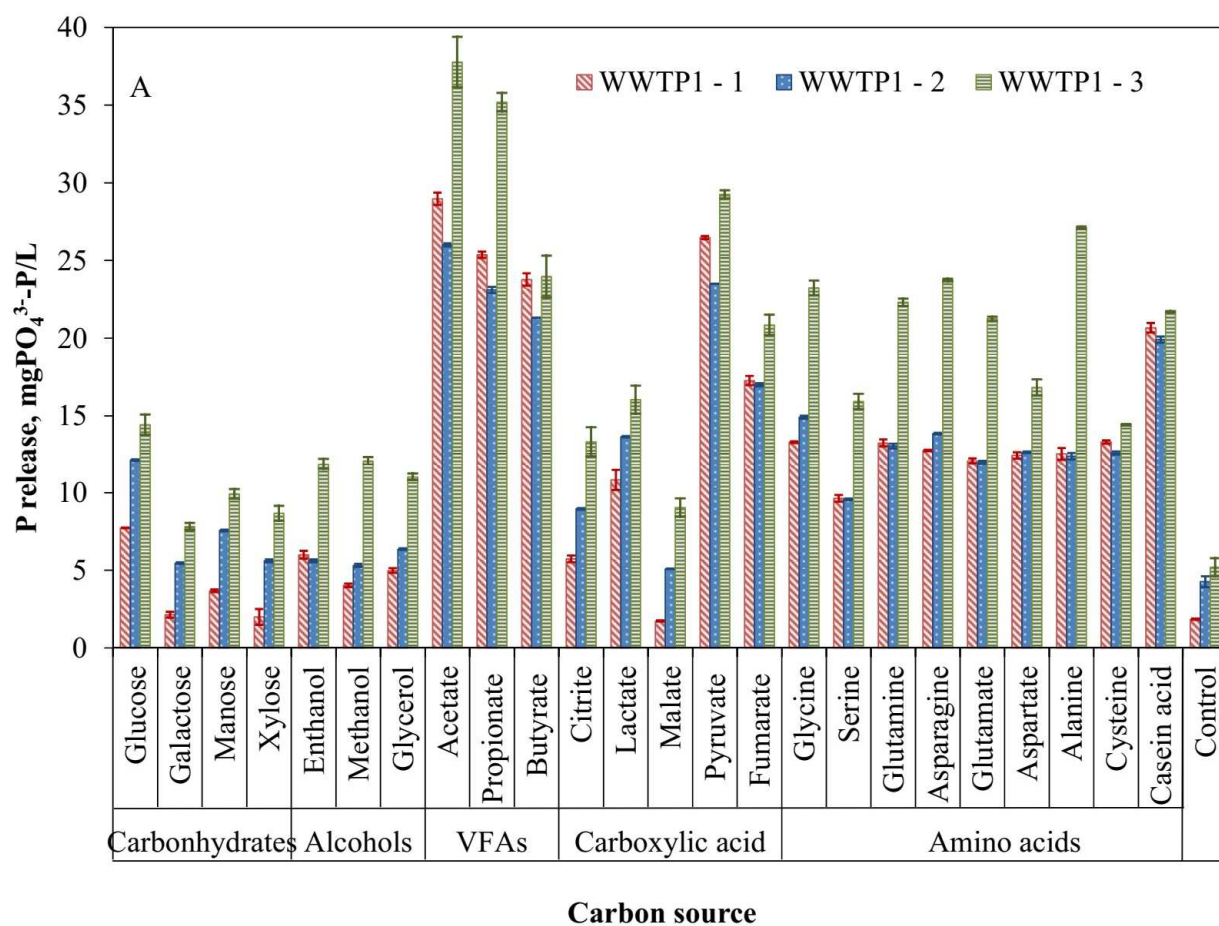
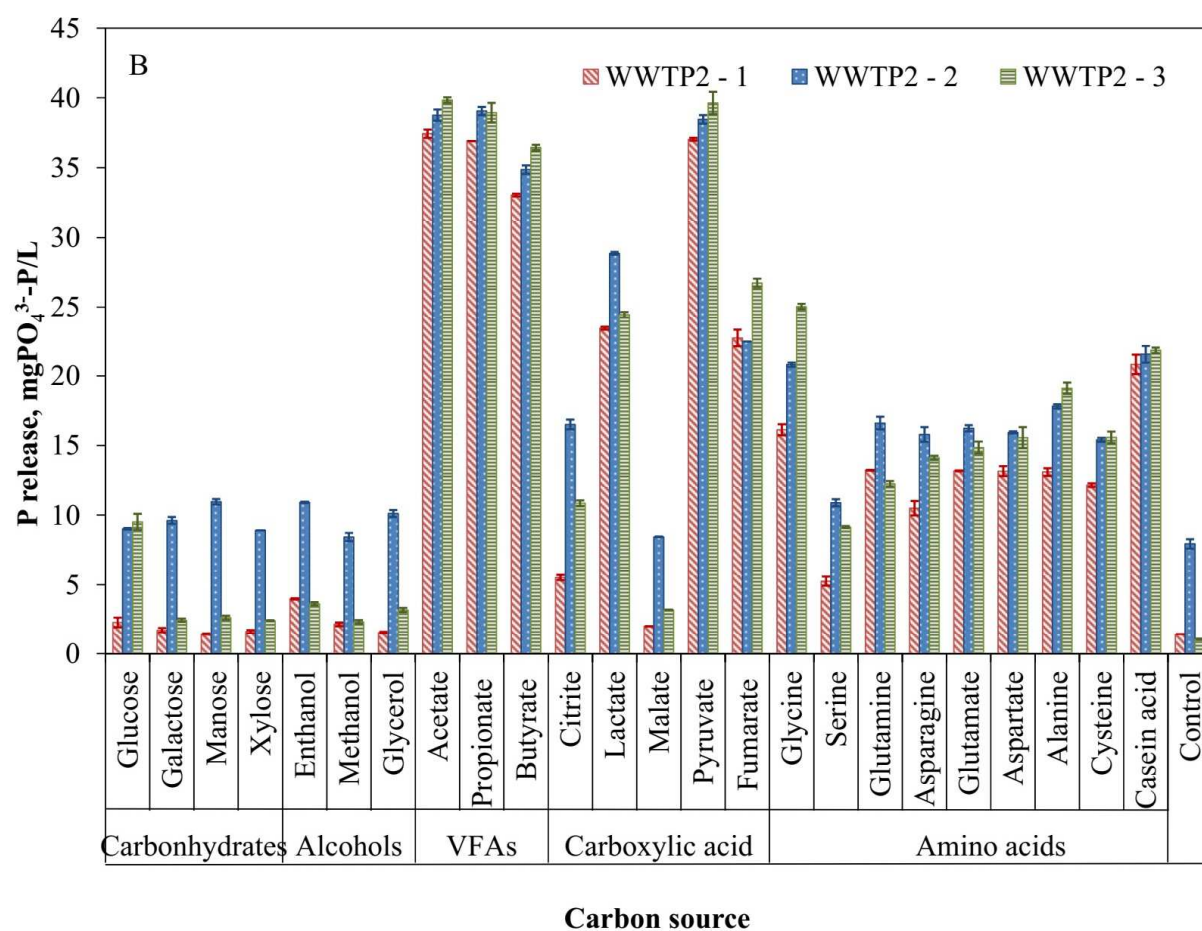
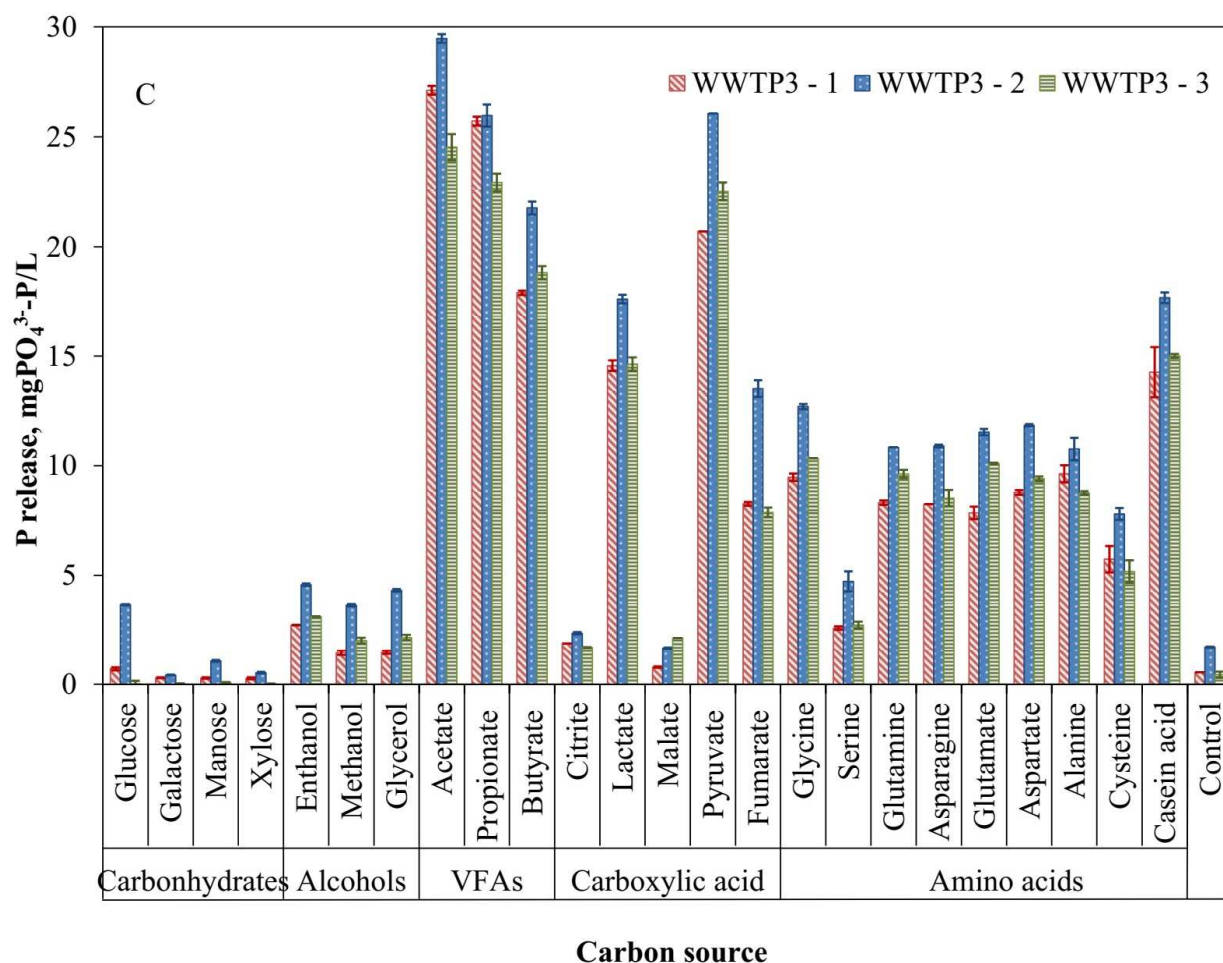


Figure 3 – Whole cell fluorescence in-situ hybridisation imaging of activated sludge from three plants. (A), WWTP1; (B), WWTP2; and (C) WWTP3. Bacteria hybridised with EUBmix (green), *Ca. Accumulibacter*–PAOs with PAOmox (red), and *Tetrasphaera*–PAOs with Tetmix (magenta).

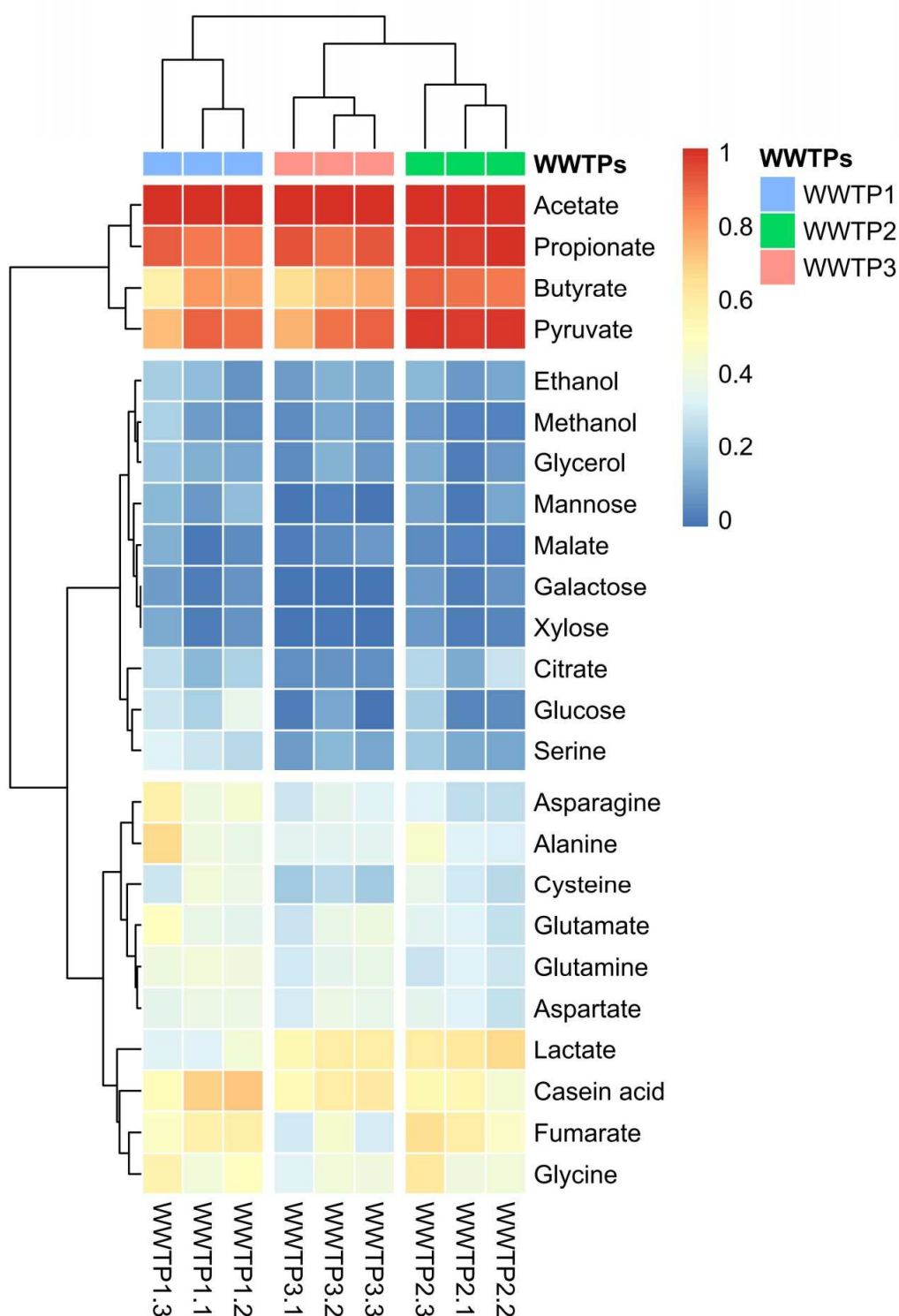






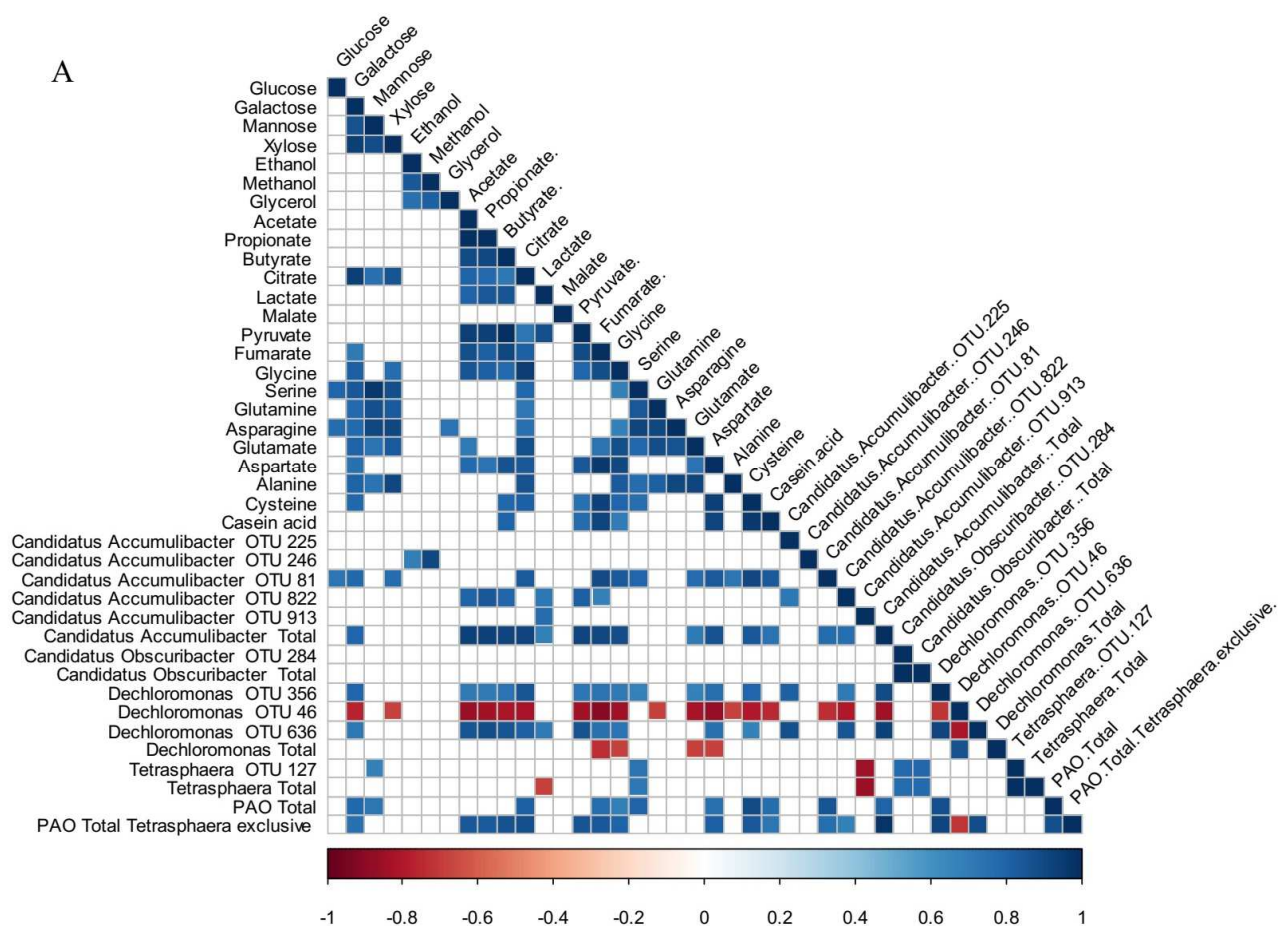
3

4 **Figure 4** – Phosphorus levels after release from activated sludge from (A), WWTP1; (B), WWTP2;
 5 and (C), WWTP3 after a 3-h anaerobic incubation with different carbon sources. Initial COD = 300
 6 mg/L, MLSS = 2.0 g/L. No carbon source was added for the control. Error bars show the range of
 7 duplicated tests.



1

Figure 5 - Heat-map with cluster analysis of the normalised P-release activities of activated sludge from three plants with different carbon sources. Values were normalised to the corresponding P-release obtained with acetate for each sampling episode.



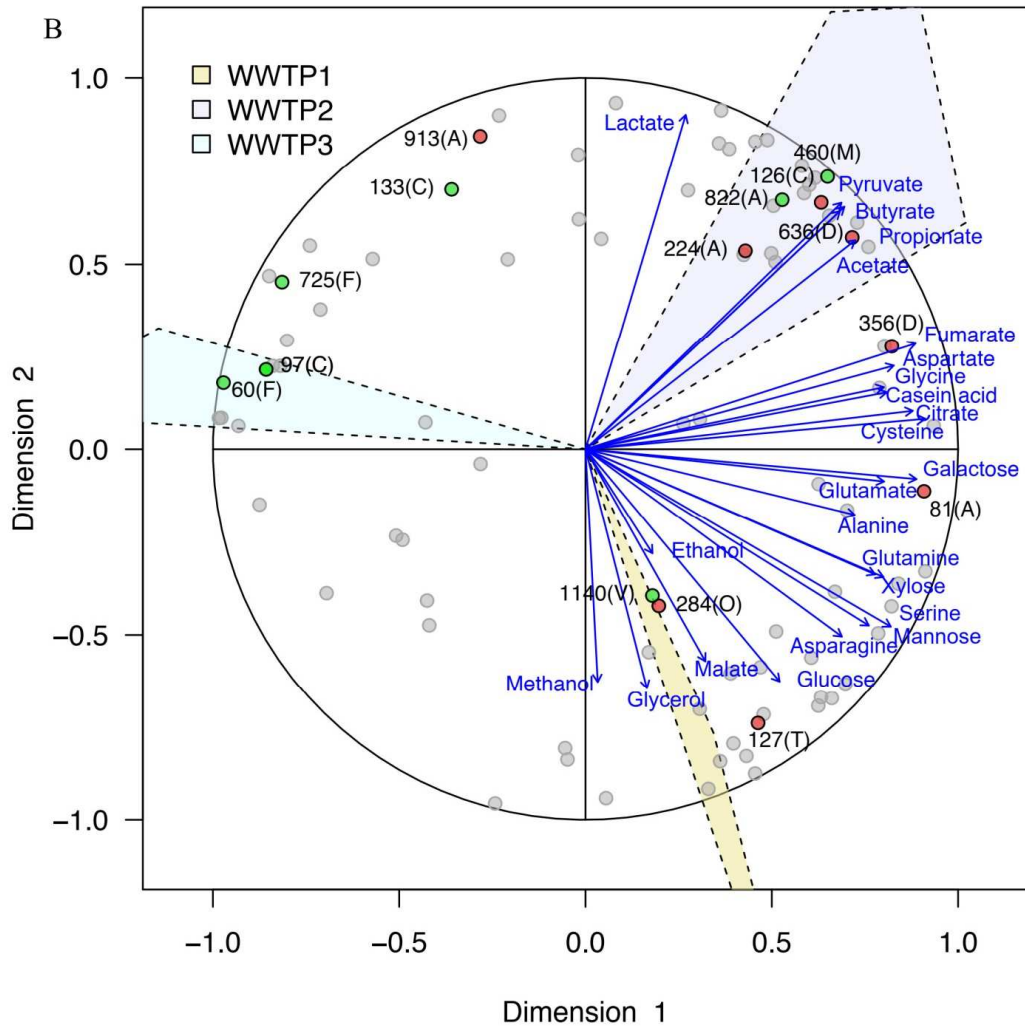
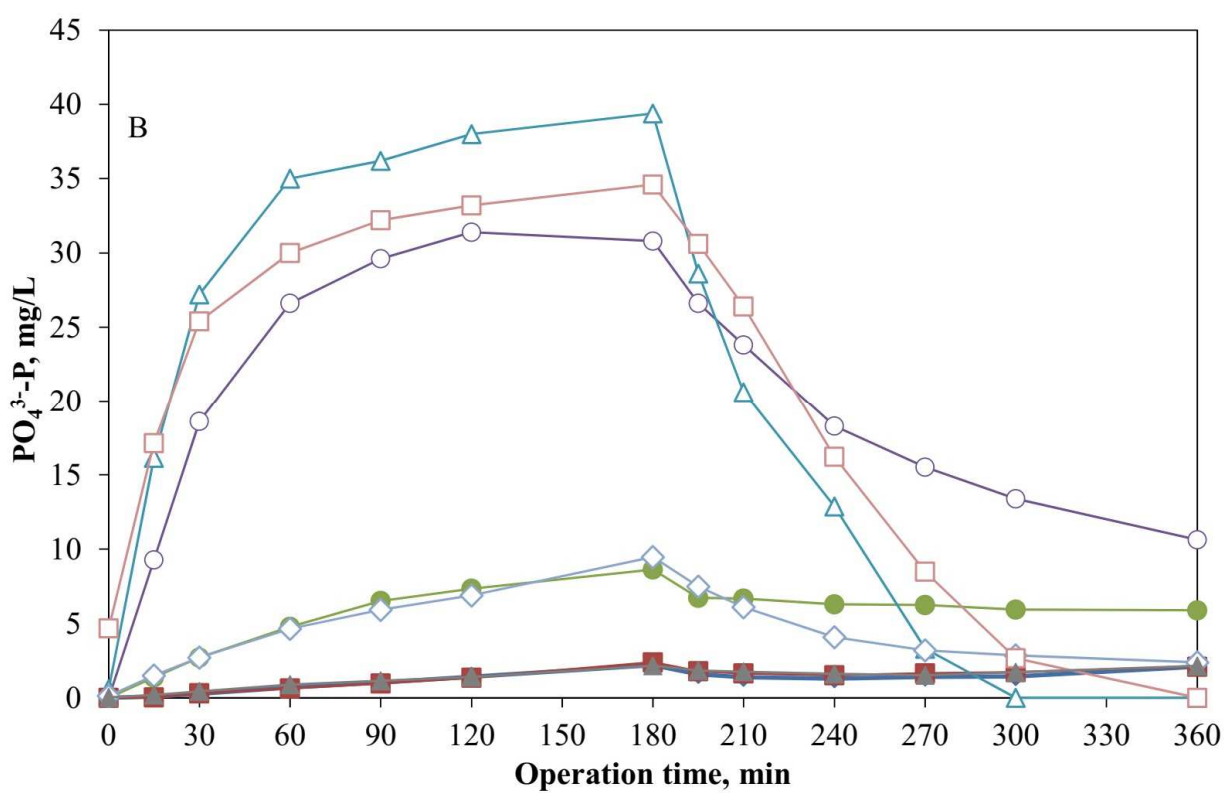
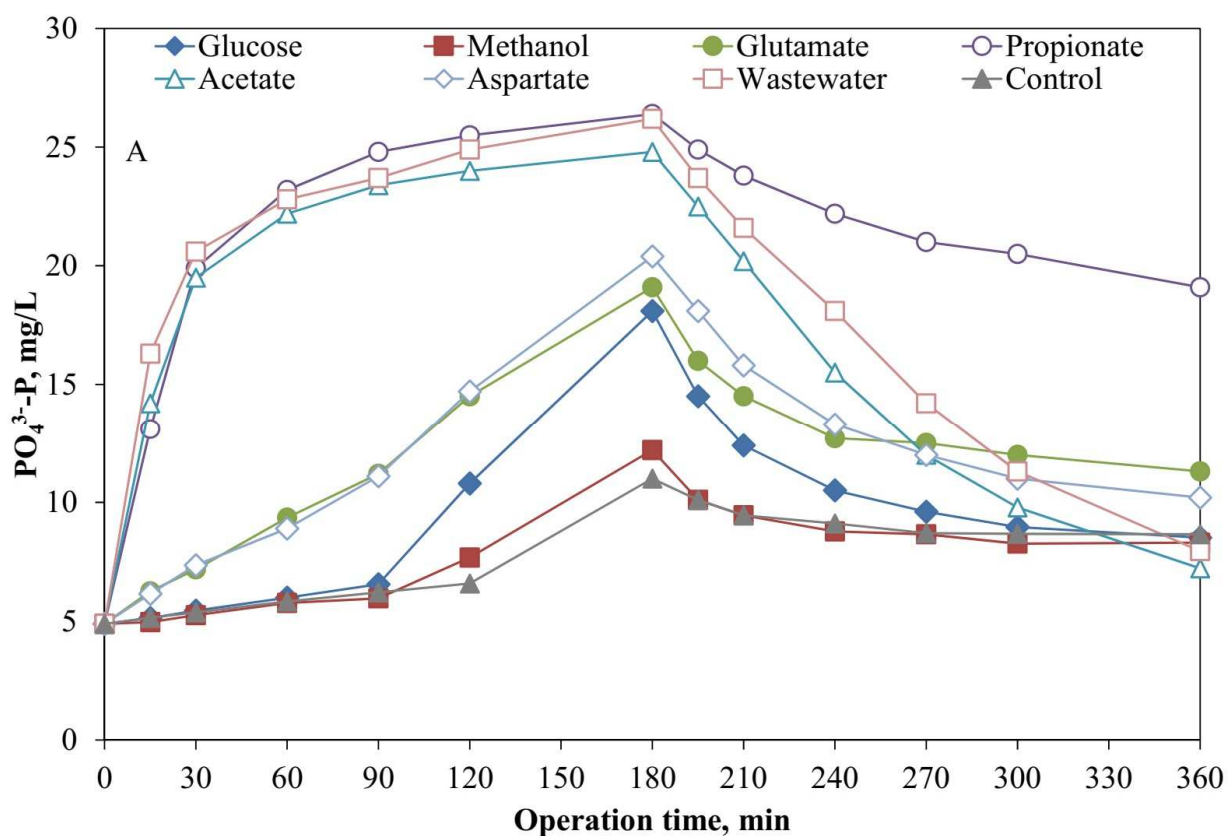
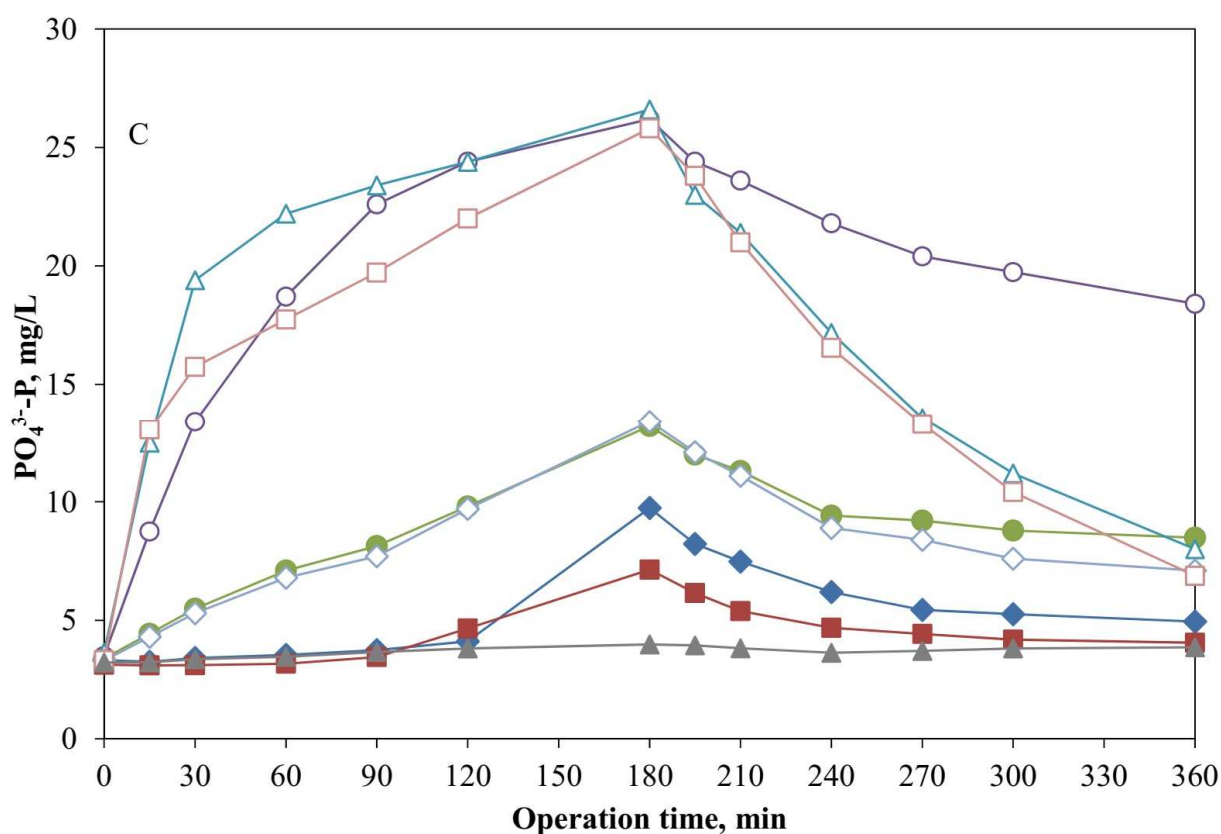


Figure 6 – Relationship between carbon utilization patterns leading to P release and abundance of specific PAOs. (A), Heatmap representation of Pearson's correlation matrix for P-release obtained with different carbon sources and the relative abundance of each group of putative PAOs (with a P-value cut-off of 0.05 and the calculated false discovery rate of 14.5%) and (B), canonical biplot of correlations between P-release profiles obtained from different carbon sources, relative abundance profiles of member taxa and sample identity based on regularised canonical correlation analysis (CCA). Correlations shown are between the 89 bacterial taxa that contain no missing values and P-release values obtained with different carbon sources. Data are projected onto the first two canonical variables. P-release variables are plotted as blue lines from the origin terminating in an arrow, and labelled with the name of the carbon source. OTU profile data are plotted with grey circles, with the

14 exception of those annotated to selected PAOs or GAOs (red and green, respectively), and which are
15 tagged with their OTU number and abbreviation ((A) *Ca. Accumulibacter*; (T) *Tetrasphaera*; (O)
16 *Ca. Obscuribacter*; (D) *Dechloromonas*; (F) *Defluviicoccus*; (C) *Ca. Competibacter*; (M)
17 *Micropruina*; (V) *Propionivibrio*. The colored convex hulls show the projections of samples from
18 each WWTP.





3

4 **Figure 7** - P-release and -uptake profiles obtained with selected carbon sources in anaerobic-aerobic
 5 cycle studies (A), WWTP1; (B), WWTP2; and (C), WWTP3. MLSS = 2.0 g/L.

Highlights

- Three full-scale tropical WWTPs in Singapore showed high in-situ EBPR activity;
- Each plant was occupied by a diverse PAO community using various carbon sources;
- *Ca. Accumulibacter* was the main active PAO at temperatures above 28°C;
- Acetate remains the most important carbon source for EBPR in tropical WWTPs;
- Carbon usage profiles were highly correlated with PAO community composition;